SERUM RETINOL BINDING PROTEIN 4 (RBP4) LEVEL IN PATIENTS WITH GESTATIONAL DIABETES MELLITUS

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BONAFIDE CERTIFICATE

This is to certify that this dissertation work entitled **" SERUM RETINOL BINDING PROTEIN 4 (RBP4) LEVEL IN PATIENTS WITH GESTATIONAL DIABETES MELLITUS"** is the original bonafide work done by **DR.R.GAYATHRI**, Post Graduate Student, Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

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CONTENTS

ABBREVIATIONS

1.	GDM	-	Gestational diabetes mellitus.
2.	RBP4	-	Retinol binding protein-4.
3.	PCOS	-	Polycystic ovarian disease
4.	HLA	-	Human Leucocyte Antigen.
5.	TNF α	-	Tumour necrosis factor alpha.
6.	TGL	-	Triglycerides
7.	HDL	-	High density lipoprotein
8.	LDL	-	Low density lipoprotein
9.	VLDL	-	Very low density lipoprotein.
10.	T2DM	-	Type 2 diabetes mellitus.
11.	ADA	-	American diabetes association.
12.	IRS	-	Insulin receptor substrate.
13.	PIP3	-	Phosphatidyl inositol triphosphate.
14.	MAP	-	Mitogen activated protein.
15.	Wnt-int	-	Wingless family.
16.	PPARG	-	Peroxisome proliferative activated receptor-gamma.
17.	PDE3B	-	Phosphodiesterase 3 B
18.	SREBP1	-	Sterol regulatory element binding protein1
19.	IGF	-	Insulin like growth factor.
20.	ROS	-	Reactive oxygen species.
21.	AGE	-	Advanced glycation end products.
22.	PKC	-	Protein kinase C.
23.	NF-κB	-	Nuclear factor kappa–B

24.	PDX	-	Pancreatic duodenal homeobox.
25.	STRA6	-	Stimulated by retinoic acid-6
26.	LCAT	-	Lecithin choline acyl transferase.
27.	LPL	-	Lipoprotein lipase.
28.	TTR	-	Transthyretin.
29.	PLA_2	-	Phospholipase A2
30.	AMPK	-	AMP dependent protein kinase.
31.	PEPCK	-	Phosphoenol pyruvate carboxy kinase
32.	NAFLD	-	Non-alcoholic fatty liver disease.
33.	HOMA-IR	-	Homeostatic model assessment for insulin resistance
34.	CRBP	-	Cytoplasmic retinol binding protein.
35.	CHE	-	Cholesterol esterase
36.	CHD	-	Cholesterol oxidase
37.	CAD	-	Coronary artery disease.
38.	ABC	-	ATP binding cassette proteins
39.	NEFA	-	Non-esterified fatty acids.
40.	CETP	-	Cholesterol ester transfer protein.
41.	RXR	-	Retinoid X receptor.
42.	SNP	-	Single nucleotide polymorphism.
43.	HNF1α	-	Hepatocyte nuclear factor 1α.
44.	JAK-STAT	-	Janus kinase-single transducer and activator of transcription.
45.	Mtor	-	Mechanistic target of rapamycin.
46.	TLR4	-	Toll like receptor-4
47.	FFA	-	Free fatty acid.

48.	IL	-	Interleukin
49.	CRP	-	C reactive protein.
50.	SCH	-	Subclinical hypothyroidism.
51.	NASH	-	Non-alcoholic steatohepatitis.
52.	Apo	-	Apoprotein.
53.	IUPAC	-	International union of pure and applied chemistry.
54.	NGSP	-	National Glucohaemoglobin Standardisation Programme.
55.	GAD	-	Glutamic acid decarboxylase.
56.	VEGF	-	Vascular Endothelial Growth factor.
57.	IAA	-	Insulin auto antibodies.
58.	TGF β	-	Transforming growth factor beta.
59.	GAG	-	Glycosaminoglycan..
60.	PDGF	-	Platelet derived growth factor..
61.	IGF	-	Insulin like Growth factor
62.	VCAM	-	Vascular cell adhesion molecule.
63.	ICAM	-	Intercellular adhesion molecule.
64.	PG	-	Prostaglandin
65.	GLUT	-	Glucose transporter.
66.	NO	-	Nitricoxide.
67.	NOS	-	Nitric oxide synthase.
68.	IFCC	-	International federation of clinical chemistry.
69.	OGTT	-	Oral glucose tolerance test.

Introduction

INTRODUCTION

Gestational Diabetes Mellitus by definition is any degree of glucose intolerance with onset or first recognition during pregnancy[1]. It is characterized by insufficient insulin levels to meet the demands in later pregnancy. The significance of GDM is because of its maternal and fetal complications like polyhydramnios, preeclampsia and overt diabetes in future, fetal complications like birth trauma, macrosomia, childhood obesity and diabetes. Apart from the Asian race to be a risk factor for GDM, the other possible risk factors are advanced maternal age, obesity, high parity, polycystic ovarian syndrome (PCOS), family history of diabetes, obstretic history of stillbirth, congenital malformation and macrosomia.

Retinol binding protein-4 is a novel marker in the pathogenesis of GDM. RBP4, a retinol transporter, plays an important role in dysregulation of insulin sensitivity in GDM.

This study has been undertaken to find the level of serum retinol binding protein- 4 in GDM and its association with lipid profile and HbA_1C has been evaluated. The raised levels also impair insulin signaling and induce gluconeogenic enzymes in the liver. Impairment of lipid metabolism is a risk factor for cardiovascular diseases.RBP4 role in lipid metabolism and metabolic syndrome has been demonstrated in various studies. There is a positive correlation

between RBP4 and LDL cholesterol, TGL and hepatic lipase activity in patients with Type 2 diabetes mellitus and cardiovascular disease.

EPIDEMIOLOGY

The prevalence of GDM in India is highly variable because of differences in living conditions, socio-economic status and dietary habits. A random survey done among the cities of India in 2002-2003, showed a prevalence of 16.55 per cent. In a study done in Tamilnadu, GDM was present in 17.8 per cent women in urban, 13.8 per cent in semi-urban and 9.9 per cent in rural areas[2]. Indian women have a 11 fold increase in risk of developing GDM, compared to the cacausians[3].

Review of Literature

REVIEW OF LITERATURE

Serum RBP4 was estimated in a group of 96 pregnant women. The values were compared between the GDM group and the control group consisting of normal pregnant women. Also RBP4 level was correlated with the patient's lipid profile and HbA_1c levels.

DIABETES MELLITUS

Diabetes mellitus is a metabolic disorder. It is characterized by chronic hyperglycemia. There is a defect in either insulin secretion or its action or both. It causes derangement of carbohydrate, lipid and protein metabolism. The chronic hyperglycemia is associated with damage and dysfunction of various organs.[4]

CLASSIFICATION OF DIABETES MELLITUS (According to ADA criteria)[5]

TYPE 1

There is β-cell destruction leading to absolute insulin deficiency.

A. Immune mediated

B. Idiopathic

TYPE 2

It ranges from predominantly insulin resistance to a predominantly secretory defect.

TYPE 3 - OTHER SPECIFIC TYPES

A. GENETIC DEFECTS OF B CELL FUNCTION

1) Maturity onset diabetes of young [MODY]

2) Neonatal diabetes

B. GENETIC DEFECT IN INSULIN ACTION

1) Lipoatropic diabetes

2) Rabson-Mendelhall syndrome

C. DISEASES OF EXOCRINE PANCREAS

1) Cystic fibrosis, 2) Hemochromatosis, 3) Fibro-calculus pancreatitis,

4) Pancreatectomy etc.

D. ENDOCRINOPATHEIS

1) Cushings syndrome, 2)Pheochromocytoma,3)Acromegaly etc.

E. DRUG OR CHEMICAL INDUCED

Thiazides, adrenergic agonists, Phenytoin, Glucoocrticoids etc.

F. INFECTIONS

Like Congenital rubella and Cytomegalovirus.

G. OTHER GENETIC SYNDROME ASSOCIATED WITH DIABETES

Down syndrome, Huntington disease, Klinefelter syndrome, Prader-Willi syndrome, Wolfram syndrome etc.

TYPE 4-Gestational diabetes mellitus

GENERAL FEATURES OF DIABETES MELLITUS

Patient presents with symptom of polyuria, polydipsia, polyphagia. It may be associated with blurred vision and susceptibility to certain infections.

INTERPRETATION OF BLOOD GLUCOSE LEVELS

FASTING BLOOD GLUCOSE	**POST PRANDIAL AFTER 2 hrs**	**DIAGNOSIS**
<100 mg/dL	<140 mg/dL	NORMAL
100 mg/dL to 125 mg/dL	140 mg/dL to 199 mg/dL	IMPAIRED GLUCOSE TOLERANCE
≥126 mg/dL	≥ 200 mg/dL	DIABETES MELLITUS

It can also result in acute life threatening complications like ketoacidosis or non-ketotic hyperosmolar coma.

Long term complications include-retinopathy, nephropathy, peripheral neuropathy, autonomic neuropathy etc.

MECHANISM OF INSULIN ACTION

Insulin is secreted by the β-cells of pancreas. It is secreted in response to glucose in the blood.

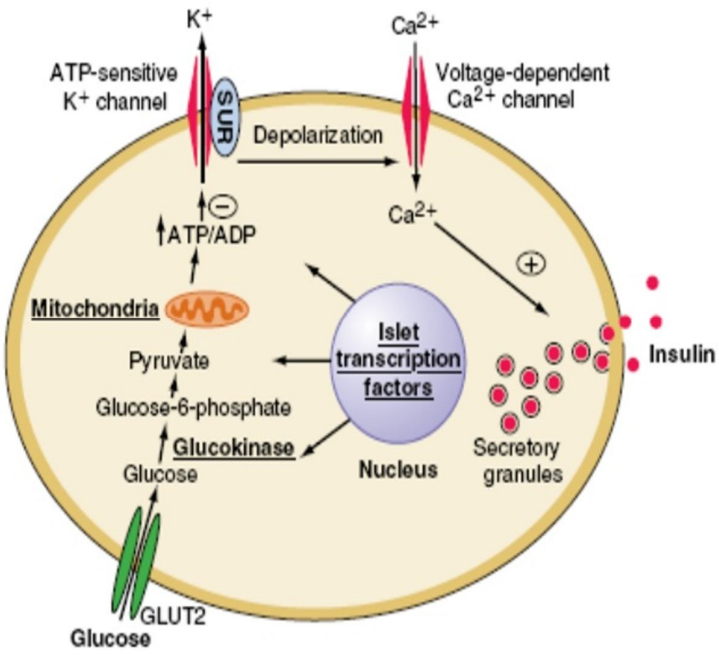

Fig-1 MECHANISM OF INSULIN SECRETION

IMAGE COURTESY: RESEARCHGATE.NET

Important stimulant of insulin secretion is blood glucose. Glucose enters the β- cells. Intracellular metabolism of glucose results in increase in ATP level. This closes the ligand gated K^+ channels, leading to depolarization of the β cell. This results in opening of Ca^{2+} channels. Calcium entry triggers the exocytosis of the secretory vescicles that contain insulin.

The biosynthesis of insulin consists of two stages-preproinsulin which is then converted to proinsulin and insulin[2]. Preproinsulin is synthesized in the endoplasmic reticulum. It is cleaved by protease to form proinsulin. The proinsulin is packed in vesicles and transported to golgi apparatus where it is cleaved to insulin and C-peptide. Insulin co-precipitates with zinc as micro

crystals within the secretary granules. Insulin is the stored as mature secretory granules.

ACTION OF INSULIN ON TARGET CELLS

Insulin binds to its receptor on the surface of the target cells. The receptor is a transmembrane glycoprotein complex consisting of two α and two β-subunits. The α subunit is entirely extracellular and carries insulin-binding site. β subunit is transmembrane protein with tyrosine kinase activity. Binding of insulin to α subunit activates the β subunit. This activates a series of proteins the Insulin Substrate Receptor protein (IRS 1-4). The second messenger PIP3 and tyrosine phoshorylated guanine nucleotide exchange proteins mediates the insulin sensitive translocation of GLUT4 from the cytosol to the plasma membrane especially in skeletal muscles and adipose tissue. Over a period of time, insulin also starts to promote the expression of genes directing synthesis of GLUT4.

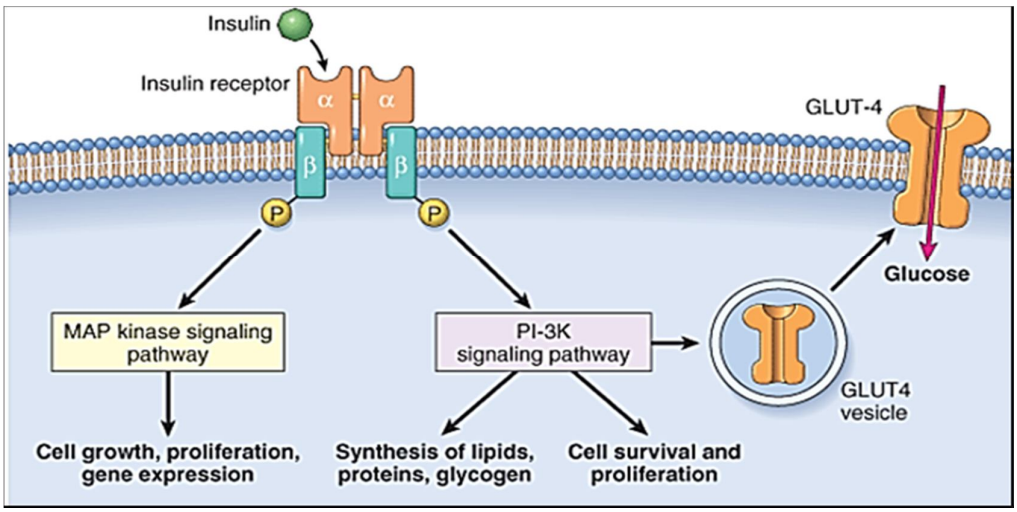

FIG 2 : ACTION OF INSULIN

IMAGE COURTESY : HARRISON'S PRINCIPLE OF INTERNAL MEDICINE

Thus the stimulation of IRS is coupled to several protein kinase signal systems:

1) Signaling through PI3-kinase and phosphatidylinositol (PI-3 kinase and protein kinase B/Akt)
2) Mitogen activated protein kinases (MAP kinases)
3) Possible interaction via kinases not coupled to IRS proteins.

MAJOR ACTIONS OF INSULIN	EFFECTS OF REDUCED INSULIN SIGNALING
Stimulates glucose transport	Increased blood glucose after meals, increased frequency of urination with loss of water glucose and electrolytes.
Control of hepatic carbohydrate metabolism	Control over gluconeogenesis and glycogen metabolism is lost.
Control of lipid metabolism in the adipocytes	Excessive release of fatty acid due to increased lipolysis

GESTATIONAL DIABETES MELLITUS

"Gestational Diabetes Mellitus by definition is any degree of glucose intolerance with onset or first recognition during pregnancy**."**

It is important to assess the trends in the prevalence of GDM, because it is associated with

- Many perinatal complications
- Offsprings are at an increased risk of developing diabetes later in life[6].
- It also helps to understand the possible mechanisms for the increase of obesity and T2DM especially in children.

PATHOPHYSIOLOGY OF GDM:

Many changes in the metabolism of mother occur in such a way as to provide sufficient energy and nutrition to the fetus. Fetus mainly depends on the maternal glucose which reaches it through the placenta, because of which the mother develops a state of "Insulin resistance" during mid-pregnancy. This state of insulin resistance progresses through the third trimester. At one stage there is reduced state of consumption of glucose by maternal tissue and increased gluconeogenesis, ensuring sufficient supply of glucose to the fetus[7].The resulting positive maternal-fetal glucose gradient, facilitates the transfer of glucose through the placenta. In a proportion of pregnancies, this state of "Insulin resistance" is greatly increased, and Gestational diabetes mellitus develops[8].

GENETIC BASIS OF GDM:

Available data suggest that there is familial tendency for GDM pathogenesis. The risk of GDM is associated with family history of T2DM[9]. They have a 2.3 fold of increased risk. Women with diabetic siblings have 8.4 fold increased risk for GDM[10].There is also a tendency for recurrence of GDM in 30%.

The defect in both insulin secretion and insulin action are explained by genetic evidence. Genetic loci of several genes responsible for insulin secretion, insulin resistance, lipid and glucose metabolism have been associated with risk of developing GDM[10].

GENES AND GENETIC VARIANTS RELATED TO INSULIN SECRETION:

1. KCJN11 GENE:

(*Potassium inwardly rectifying channel, subfamily J,member 11)*

This gene encodes for the ATP-sensitive potassium channel of β cells. A variant of this, results due to substitution of lysine for glutamic acid, Glu23Lys. This variant induces over-activity of the potassium channels, thus decreasing the insulin secretion[11].The risk is 6.2% for *KCNJ11* Lys23/Lys23 genotype and 10.1% for *KCNJ11* Glu23/Lys23 and Lys23/Lys23 combined[12].

2. TCF7L2(Transcription factor 7-like 2):

It is involved in gene transcription. It is a member of the Wnt signaling pathway. The strong association of variants of TCF7L2 and GDM is through the mechanism involving insulin secretion[13,14]. It is found that T allele of the rs7903146 variant[15] and T allele of rs12255372 variant[16] were frequently associated with GDM.

3. Mitochondrially encoded NADH dehydrogenase 1(ND1):

It is part of electron transport chain and involved in glucose metabolism. Reduced activity of the respiratory chain and decreased production of ATP result insulin secretion defects[17]. Carriers of Thr31 allele of the Met31Thr polymorphism are associated with GDM[18]. These women also have a positive maternal history.

INSULIN SIGNALING GENES

(1) Insulin receptor (INSR):

Insulin receptors are involved in glucose uptake. Thus insulin receptor genes are involved in glucose homeostasis. Mutations in INSR causes severe insulin resistance[19].INSR polymorphism has been associated with GDM[20].Polymorphism of INSR KPN1 was associated with GDM[21].

(2) Insulin receptor substrate 1(IRS 1):

It is a substrate of insulin receptor tyrosine kinase. It is the key participant in insulin signaling[22]. The Gly972Arg polymorphism reduces tyrosine phosphorylation and inhibits insulin receptor kinase, thus resulting in insulin resistance[23]. The 972Arg variant has 25% greater risk of developing T2DM[24].The homozygous Arg972 was associated with GDM[25].

LIPID AND GLUCOSE METABOLISM GENES:

Peroxisome proliferative activated receptor-gamma (PPARG) :

PPARG is a transcription factor that regulates adipocytes differentiation and also in lipid and glucose metabolism. It binds to specific response elements in promoter regions of target genes. A variant of PPARG has been identified and involves the substitution of alanine instead of proline at 12^{th} amino-acid position (Pro12Ala). This alanine 12 variant decreases the promoter element affinity of PPARG. This has been shown to increase the risk of T2DM as well as GDM by decreasing insulin sensitivity.

OTHER GENES

MANNOSE BINDING LECTIN PROTEIN C 2(MBL2):

MBL2 is a membrane of the collectin family of proteins. It is a component of the innate immune system. It also influences inflammatory response by inhibiting TNFα. Its deficiency is associated with recurrent infections and chronic inflammatory diseases.

Two variants of MBL2 gene-Arg52Cys and Gly54Asp are associated with decreased plasma MBL2 level. Of these, the carriers of Asp54 allele of Gly54Asp polymorphism have been shown to be associated with GDM. These polymorphisms are also shown to be linked to micro and macrovascular complications associated with diabetes.

METABOLIC IMPLICATIONS OF GDM

CARBOHYDRATE METABOLISM

In early pregnancy, the blood glucose and insulin levels are almost close to the non-pregnant state. Peripheral insulin sensitivity and basal hepatic output are normal in early trimester[26].

But in third trimester, though there is

- Increased insulin production, the insulin action decreases by 50-70% in normal pregnancy[27].
- The postprandial blood glucose concentration is significantly elevated and the glucose peak is prolonged[28].

- Basal hepatic glucose output is increased by 16-30% to meet the needs of the growing fetus[29].

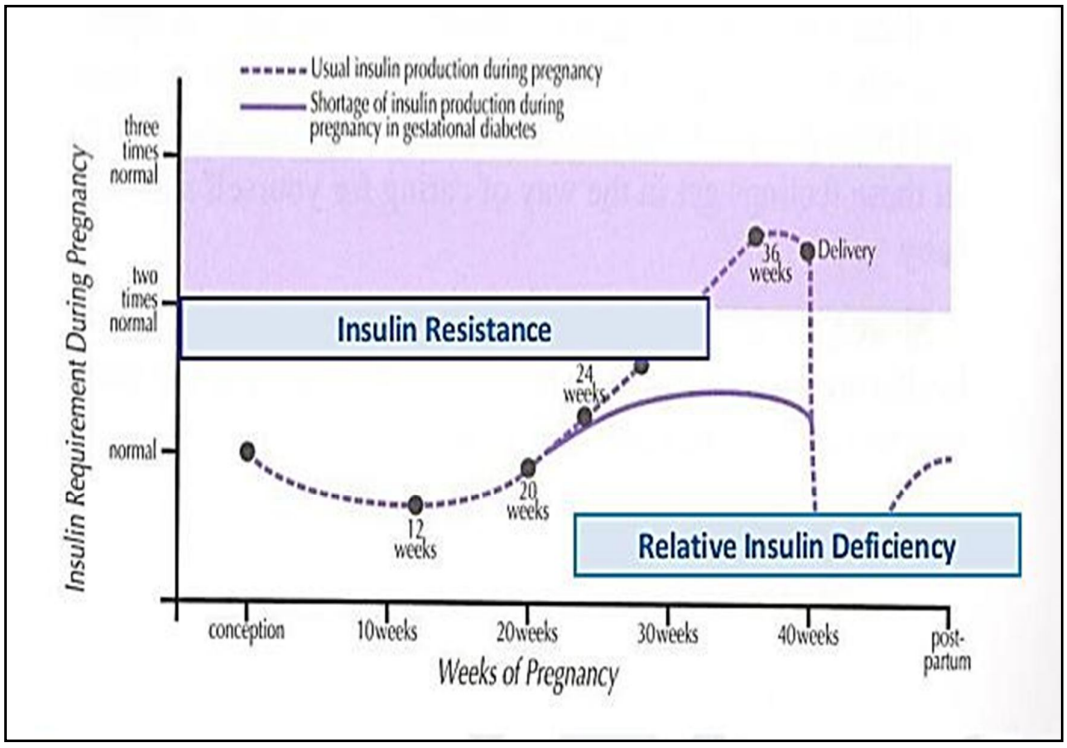

FIG 3:PATHOPHYSIOLOGY OF GDM

IMAGE COURTESY:UEDAEGYPT.ORG

Inspite of the anti-insulinogenic action of the placental hormones, carbohydrates acts as the main source of energy for the pregnant women. So the fasting glucose level remains low[30].

In GDM, there is decrease in first-phase insulin response. Normally about 80% of glucose in postprandial state is stored in the muscle as glycogen[31].But, because of the reduced insulin sensitivity and defective transport mechanism, the muscle glucose entry and thence the glycogen production is reduced.

In the liver normally, hyperglycemia primarily inhibits net hepatic glycogenolysis by inhibiting glycogen phosphorylase, and hyperinsulinemia stimulates glycogen synthase. But these processes are suppressed in GDM[32]. The hepatic glucose output is not suppressed by insulin in later pregnancy[33].This results in hyperglycemia.

LIPID METABOLISM

Increased estrogen, progesterone and insulin favor lipid deposition in early pregnancy. In later pregnancy, there is lipolysis and fat mobilization[34]. Thus there is a change from anabolic to catabolic state. This promotes the use of fatty acids as energy source for the mother and reserving glucose and aminoacids for the fetus.

Insulin action on mature adipocyte is to enhance glucose uptake and its storage as triglyceride. Insulin also enhances fatty acid uptake by adipose tissue, by stimulating lipoprotein lipase activity.

INSULIN

⇩

Stimulate phosphodiesterase 3B (PDE3B)

⇩

↓Intracellular cAMP

⇩

Attenuates protein kinase

⇩

Inactivation of hormone sensitive lipase

⇩

Inhibits lipolysis

In pregnancy,

- Increased free fatty acid and glycerol concentration.
- Decrease in glucose, alanine and β-hydroxybutyrate
- Increase in triacylglycerol, fatty acids, phospholipids and cholesterol [32].
- Clearance of VLDL is reduced because of decreased activity of lipoprotein lipase.
- HDL cholesterol level increases by 12 weeks and remains elevated throughout pregnancy[34].

GDM induces a state of dyslipidemia consistent with insulin resistance. Women with GDM have higher triglyceride levels. There is reduced oxidation of exogenous triacylglycerol. The increased triacylglycerol is due to

(1) reduced fatty acid uptake and its subsequent oxidation

(2) increased hepatic oxidation and esterification of fatty acid thus increasing the synthesis of VLDL[37].

INSULIN RESISTANCE IN SKELETAL MUSCLE AND ADIPOSE TISSUE:

Muscle insulin resistance promotes hepatic steatosis by inducing *SREBP1c* mediated lipogenesis and inhibition of fatty acid oxidation[38].

In late pregnancy, postprandial free fatty acid level increases and the disposal of blood glucose by insulin worsens by 40-60%, when compared with pre-pregnancy status[46].

Skeletal muscle is the principle site of glucose disposal in the body. But this becomes severely insulin resistant along with the adipose tissue during later half of pregnancy. Normal pregnancy itself is characterized by decrease in insulin mediated glucose disposal by 50%. In order to maintain the euglycemic state, insulin secretion is increased by 200-250%[47].Placental derived hormones, especially Human Placental Lactogen (hPL) and Human Placental Growth hormone (hPGH) are believed to re-programme the maternal physiology, resulting in an insulin resistant state[48].

EFFECTS OF MATERNAL HORMONES:

1) HUMAN PLACENTAL LACTOGEN(hPL):

Human placental lactogen is the product of *hPL –A* and *hPL-B* genes secreted into both maternal and fetal circulation after 6 weeks of gestation[40].

Its level is decreased in hyperglycemia and elevated in hypoglycemia. Its metabolic role is to mobilize lipids and free fatty acids[41]. During second half of pregnancy, the hPL level increases by 10 fold. It stimulates lipolysis and increases free fatty acid level. Thus it provides a different fuel for mother so that glucose and amino acids are conserved for the fetus.

The increased fatty acid level in turn interferes with insulin directed entry of glucose into the cell. Thus hPL is considered as a potent antagonist to insulin action during pregnancy[42].

PLACENTAL GROWTH HORMONE (PGH):

Produced by syncytiotrophoblast cells of human placenta. It is the product of the *GH-V* gene. It is predominantly found in maternal circulation. Its concentration increases progressively from mid-pregnancy[43].It is an important potential regulator of maternal insulin resistance during pregnancy. It mainly influences the fetal growth by modifying substrate availability[44].It influences the p85 expression of PI 3 kinase activity in skeletal muscle, thus contributing to insulin resistance in pregnancy[45].

Furthermore, hPl and PGH stimulate insulin-like growth factor (IGF) production and modulate intermediary metabolism, resulting in increase in the glucose availability to the fetus[49].

MECHANISMS OF INSULIN RESISTANCE:

"When the biological effects of insulin are less than expected for glucose disposal in skeletal muscle and adipose tissue and suppression of endogenous glucose production primarily in the liver, it is said to be insulin resistance."

Insulin resistance may be due to a decrease in the number of insulin receptors or impairment in post-receptor signaling of insulin receptors.

GLUCOSE TOXICITY:

Glucose in chronic excess causes damage to the structure and functions of the organs. Various pathways have been suggested for metabolic consequences of excess glucose. All these pathways have in common the formation of reactive oxygen species. This causes chronic oxidative stress resulting in defective insulin gene expression and its secretion.

1) GLYCERALDEHYDE AUTOXIDATION:

Glyceraldehyde 3-phosphate is a phosphorylation product formed from glucose during glycolysis. Autoxidation of hydroxyaldehydes generates hydrogen peroxide and ketoaldehydes[50].

Hydrogen peroxide forms toxic hydroxyl radical in the presence of redox active metals. So, this process contributes to two potentially toxic substances:

(a) ketoaldehydes- which contributes to glycosylation-related protein development and

(b) the hydroxyl radical, which can cause mutagenic alterations in DNA.

Excess glyceraldehydes inhibits insulin[51].

Long term high glucose level decreases Glyceraldehyde 3-phosphate dehydrogenase(GAPDH) activity,through the ROS-activated poly ADP ribosylation[52]. This in turn is associated with intracellular advanced glycation end product (AGE) .

2) PKC ACTIVATION*:*

Dihydroxyacetone undergoes reduction to glyceraldehydes 3-phosphate and acylation, thus increases de novo synthesis of diacylglycerol, which activates protein kinase C. Activation of PKC is associated with increase in TGF-1, vascular endothelial growth factor, NADPH-oxidase, NF-B and ROS[53,54].

3) METHYLGLYOXAL AND GLYCATION:

These 3 molecules are reactive intracellular dicarbonyls. They form Advanced Glycation End products by reacting with amino group of intra and extracellular proteins[55]. AGEs play important role in pathogenesis of microvascular complications of diabetes[56].

High blood glucose

Impaired GADPH activity

Accumulation of glyceraldehydes 3-P & DHAP

↑ Methylglyoxal

Hyperglycemia leads to the formation of Advanced glycosylation End (AGE) products by Non-enzymatic glycosylation of intracellular proteins amino group with these dicarbonyl molecules. These glycated proteins undergo progressive dehydration, cyclization and oxidation.

4) POLYOL PATHWAY:

Hyperglycemia increases glucose metabolism via sorbitol pathway. Glucose is converted to sorbitol by the enzyme aldose reductase.

- It causes, reduced cytosolic NADPH which in turn increases oxidative stress in the cell.
- Increase cytosolic ratio of NADH/NAD+ causes inhibition of the enzyme glyceraldehydes 3-phosphate dehydrogenase
- Formation of methlglyoxal.
- Increased AGE production.

This pathway is normally inactive and gets activated only when intracellular glucose level increases.

5) INCREASED HEXOSAMINE PATHWAY:

Fructose 6-phosphate is diverted from glycolysis to form glucosamine 6 phosphate, which in turn is converted to UDP-N-Acetyl glucosamine. N-Acetyl glucosamine supports proteoglycan synthesis and also formation of *O*-linked glycoproteins. They can glycate transcription factors and thus enhance transcription of gene including plasminogen activator inhibitor.

EFFECTS OF CHRONIC OXIDATIVE STRESS ON INSULIN GENE EXPRESSION:

Chronic exposure of the beta cell to high concentrations of glucose causes defective insulin gene expression as well as marked decrease in insulin secretion[57].

The defect in insulin gene expression is due to loss of atleast two critical proteins that activate the insulin promoter. One is Pancreatic duodenal homeobox-1(PDX-1)[58,59]and the other is MafA[60,61]. MafA is RIPE -3b1 activator.

Due to chronic oxidative stress, the DNA binding capacity of PDX-1 is reduced[62-64]. Also the transcriptional activity of Glucokinase enzyme is reduced.

RELATIONSHIP BETWEEN GLUCOSE TOXICITY AND LIPOTOXICITY:

Diabetes mellitus is often accompanied by raised levels of cholesterol, free fatty acids and triglycerides[65].

It has been reported that prolonged exposure of pancreatic β-cells to fatty acids, inhibit insulin gene expression[66].Simultaneous presence of hyperglycemia and elevated fatty acids cause accumulation of cytosolic citrate, the precursor of malonyl-CoA, which in-turn inhibits carnitine palmitoyl-transferase 1(the enzyme required for fatty acid transport into the mitochondria)[67].

In the presence of high glucose concentrations, elevated fatty acids are not readily oxidized in mitochondria but are shunted towards esterification pathways. It has been observed that adverse effects of palmitate on insulin gene expression and secretion was seen only when β-cells were simultaneously exposed to high concentrations of glucose. Palmitate induced accumulation of beta cell triglycerides occurred only in the presence of high glucose[68]. Thus lipotoxicity requires antecedent hyperglycemia[69].

DIAGNOSIS OF GDM.

SCREENING TESTS:

A screening test can be performed on either a selective or on universal basis. Women with positive screening test result are subjected to a diagnostic test, which is more extensive and demanding.

Generally, screening and diagnostic tests for GDM are performed between 24 to 28 weeks of gestation, because at this point in gestation the diabetogenic effect of pregnancy manifests and there is sufficient time to intervene[70]. A good screening test has a positive likelihood ratio of atleast 6. Screening on the basis of risk factors seems to be inefficient[71]. In other words, a diagnostic test only in women with risk factors will miss many women with GDM.

GLUCOSE CHALLENGE TEST (GCT):

Patient is given 50 grams glucose solution irrespective of food intake[72,73]. Blood glucose measurement is taken either after 1 or 2 hours, each having different cutoff values.

The American Diabetes Association (ADA) recommends a cutoff value of 140mg/dL after 1 hr which identifies 80% of women with GDM or 130 mg/dL which identifies 90% cases[74].

The 2 hours cutoff is 118 mg/dL.

Patients with positive GCT are subjected to diagnostic test.

The limitation of this test is that it has many false-positives and sensitivity is only 86%[75].

OTHER TESTS:

Capillary blood glucose measurement with hemocue	Mainly depends on the meter and not reliable.
Breakfast test and lunch test	Needs to use standard diet Not commonly used
Glycosuria	Not reliable in GDM
Fructosamine	Less sensitive
Glycated hemoglobin	Less sensitive in pregnancy Not reliable.
Fetal abdominal circumference (AC)	Misses 43% of GDM cases.

DIAGNOSTIC TEST :

"The gold standard for diagnosis of GDM is the *100 gm 3 hour Oral Glucose Tolerance test (OGTT)"*.

Patient preparation includes

1) Patient must take atleast 150 gm of carbohydrate per day for the preceding 3 days.
2) Fasting between 10 to 16 hrs of fasting
3) Patient must be at rest for 30 minutes before blood collection.
4) No smoking is allowed.

PROCEDURE:

- Fasting blood and urine samples are collected.
- After the first measurement, the patient drinks 100 gm glucose solution with 300 ml water within 5 minutes.
- One, 2 and 3 hours blood and urine samples are taken.

- During the procedure, the patient is not allowed to smoke or walk.
- But the WHO recommends to use a low glucose load of only 75 gms. Only fasting blood glucose measurement and one measurement 2 hrs after the glucose load is taken. The interpretation of these test results are shown in table.

	Carpenter and Coustan /ADA (2004)		**WHO (2008)**	**IASPSG / ADA (2012)**
	75 gm OGTT	**100 gm OGTT**	**75 gm OGTT**	**75 gm OGTT**
Fasting	95 mg/dL	95 mg/dL	126 mg/dL	92 mg/dL
1 hr	180 mg/dL	180 mg/dL		180 mg/dL
2 hr	155 mg/dL	155 mg/dL	140 mg/dL	153 mg/dL
3 hr		140 mg/dL		

Citation : Cheung KW, Wong SF (2012) Gestational Diabetes Mellitus update and review of literature, Reproductive Sys Sexual Disorder S2:003.

COMPLICATIONS OF GDM:

It includes both maternal and fetal complications.

1) FETAL MACROSOMIA:

The incidence of fetal macrosomia is 6 times more common in GDM than in normal pregnancies[76]. The cutoff value for birth weight above the 90th percentile[77] or birth weight of more than 4000 gms for macrosomia[78].The reported incidence of macrosomia in women with GDM is 16%-29% when compared to normal pregnancy with a incidence of 10%[79,80].

Mother's age, pre-gestational weight, weight gain during pregnancy, fetal insulin secretion and substrate concentration in maternal circulation are the main factors which influence the fetal weight[81].

Maternal glucose passes through the placenta to reach the fetus, which stimulates fetal insulin secretion. Insulin, being an anabolic hormone increases fetal dimension causing macrosomia[82]. Significantly elevated levels of triglycerides in cord blood of obese GDM women fetuses with macrosomic babies suggest that triglyceride plays an important role in pathogenesis of macrosomia[83,84] . There is reduced activity of lipoprotein lipase in GDM due to insulin resistance. So there is reduced lipolysis of TGs and decreased suppression of endogenous TGs synthesis[85].

2) SHOULDER DYSTOCIA AND BIRTH TRAUMA:

The increase in birth trauma in the offsprings of mother with GDM is due to higher rate of macrosomia, which predisposes to shoulder dystocia.

Shoulder dystocia leads to consequences like clavicular fracture and brachial plexus injury[72]. A planned caesarean could avoid such complications[86].

3) CAESAREAN DELIVERY:

Macrosomia is only an intermediate outcome, which is not damaging by itself. But, macrosomia leads to various consequences like caesarean deliveries, instrumental deliveries (forceps and ventouse deliveries)birth trauma including

brachial plexus injury or neonatal hypoglycemia. An early diagnosis and treatment of GDM can avoid such consequences.

4) NEONATAL METABOLIC PROBLEMS:

Due to GDM, an increased incidence of [87]

- neonatal hypoglycemia,
- hypocalcemia,
- hyperbilirubinemia and
- polycythemia has been reported.

This causes unnecessary admissions in Neonatal Intensive Care unit (NICU). Neonatal hypoglycemia is much more related to macrosomia, than to maternal GDM[88]. Also the long term damage due to neonatal hypoglycemia is seen only in those with hypoglycemic seizures or recurrent hypoglycemia[89]. Adequately treated hyperbilirubinemia has no lasting effects on the infant[90].The other outcomes include, inconvenience for the baby being subjected to repeated blood tests, costs of these tests, separation of mother and the neonate due to NICU admission.

5) HYPERTENSION/PREECLAMPSIA:

GDM has been associated with hypertension, but its association is inconsistent. In one study, it has been shown that the incidence of hypertension in GDM is 20% more when compared to normal controls[91]. But both of these conditions are suggested to be related to maternal age and BMI.

6) LATE EFFECTS OF GDM ON MOTHER AND BABY:

The prevalence of T2DM is higher, when compared to those without GDM in earlier pregnancies[92]. The prevalence of T2DM later in life for women with GDM varies from 9% in Causcasians to 25% in Asian women[93,94].Children of mother with GDM are also at increased risk of developing T2DM and obesity in later life[95]. They are also at a risk of developing neuropsychological problems[96].

RETNOL BINDING PROTEIN-4

- Serum Retinol binding protein-4 is a specific carrier protein which belongs to lipocalin family, of kernel type, calycin superfamily.
- It is coded by chromosome no: 10. Its location is 10q23.33 in cytosol.
- The protein weighs 23 kDa and has 201 amino acids.
- It consists of 4 chains which are linked by 3 disulphide bonds.
- It is a monomer.
- It undergoes methylation as a post translational modification.
- It is secreted by hepatocytes and adipocytes.
- It is the transporter of Retinol (Active form of vitamin A) from the liver to the peripheral tissues.
- It is bound to transthyretin (prealbumin), which is a carrier of thyroid hormone.
- Lipocalin protein family are small secreted proteins, which are characterized by
- their ability to bind to small hydrophobic molecules like retinol

- binding to specific cell surface receptors.
- formation of macromolecular complexes.
  - They exhibit great structural and functional diversity. They have been shown to be involved in
  - retinol transport
  - pheromone transport
  - prostaglandin synthesis
  - cell growth and metabolism modulation
  - immune response regulation
  - tissue development

STRUCTURE[97]:

The members of this family share about 20% sequence homology, but mainly confined to a common tertiary structure determined by conserved segments of the individual lipocalin protein, called the lipocalin folds.

These folds organize the lipocalins in eight anti-parallel β- sheets surrounding a hydrophobic pocket. This hydrophobic pocket is essential for functioning as carrier proteins.

7 short loops are present and named as L1 to L7. They connect one β-sheet with other. When ligand binds to the protein, it forms a cavity and is closed by loop L1, which acts as a lid.

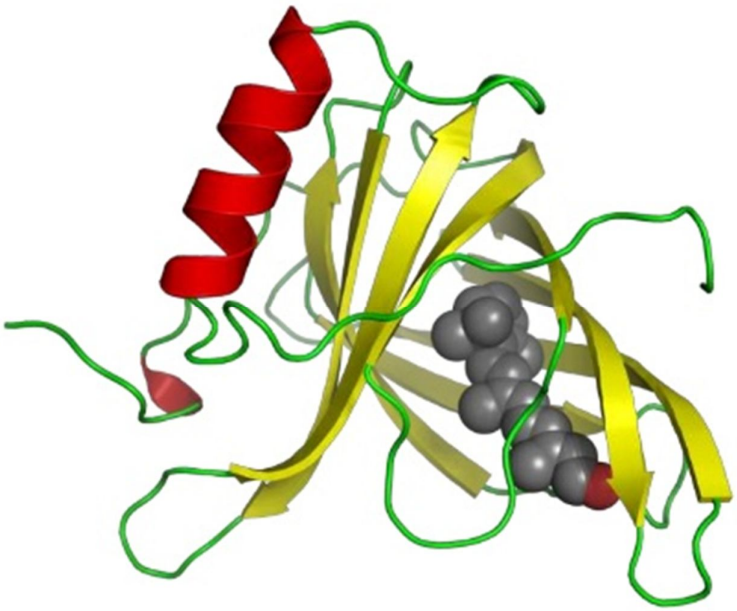

FIG 4: STRUCTURE OF RBP4 PROTEIN WITH TRANSTHYRETIN

IMAGE COURTESY:en.wikipedia.org

Additionally, there are 3 to 10 helices at the amino terminus, outside the β-barrel and an α-helix at the carboxy terminus.

RBP4-TRANSTHYRETIN COMPLEX[97]:

In plasma, RBP4 is complexed to transthyretin by a non covalent linkage. It combines with transthyretin in equimolar concentration in 1: 1 ratio. It is bound to transthyretin to avoid excretion by the renal glomeruli.

Only 4% of RBP is free, the rest is bound to transthyretin[98]. Transthyretin has greater affinity for holo-RBP than apoRBP. The complex is stable between pH 5.0 and 9.0 and dissociates at low ionic strength.

The interaction of the proteins to form macromolecular complex is mediated by the loop scaffold. The loop scaffold forms the open end of the

molecule. The variation in the length of the loop, its conformation and amino acid composition is specific, which binds with high affinity to transthyretin.

RBP4 RECEPTOR[100,101]:

RBP4 has two receptors namely

1) Stimulated by Retinoic Acid 6(STRA6)
2) RBP4 Receptor 2(RBPR2)

The cellular receptor for RBP was identified and biochemically characterized as the protein named STRA6 (Stimulated by Retinoic acid 6)[99]. It is a 75-kDa multipass transmembrane protein. It has 9 transmembrane segments.

STRA6 receptors are expressed in several tissues, including retina,brain, testis, muscle and placental endothelial cells. It is not found in the liver and adipose tissue.

RBPR2 (RBP Receptor 2) is another receptor found in the liver and adipose tissue. It is a 70.1kDa protein. It is also a transmembrane receptor with more than 50% similarity in its amino acid sequence with the STRA6 receptors. Both the receptors have similar intracellular C-terminal soluble domains containing 74-75 amino acids. But RBPR2 lacks the conical Src homology 2 domain of STRA6 C terminus. RBPR2 mediate the retinol recycling from plasma to the liver.

STRA6 catalyses the release of retinol from RBP, where it is tightly bound. This facilitates the translocation of retinol across the membrane and its association with the cytoplasmic CRBP1 receptors.

Conversion of retinol to retinyl esters for storage by the enzyme lecithin retinol acyl transferase (LRAT), increases the amount of CRBP1 protein, thereby enhancing the action of STRA6 receptors[99].

BIOCHEMICAL FUNCTIONS OF RBP4:

It is an extracellular transport protein. RBP is the sole retinol transporter in the plasma. It binds to a single all-*trans*-retinol molecule. The apo-protein is saturated with retinol, which triggers its secretion into the plasma.

Its physiological functions are[97]

1) It facilitates transport of insoluble retinol from storage site to the peripheral tissues
2) RBP protects bound retinol from oxidation.
3) The synthesis of RBP regulates release of retinol from the liver and mediates specific uptake by target tissue.

MECHANISM OF ACTION ON TARGET CELLS[101]:

Retinol is taken by the RBP protein in the plasma. This holo-protein attaches to the cell surface of the target cell by the STRA6 receptor. Due to hydrophobic interaction of retinol and the STRA6 outer cleft, the binding of RBP

to the receptor weakens its affinity for retinol leading to its delivery to the outer cleft of the receptor.

Inside the cell, the retinol is delivered to the CRBP-1(Cytoplasmic retinol binding protein 1). There is unidirectional movement of retinol from RBP to CRBP1. As per the body requirement, retinol is converted to retinoic acid which is carried by cytoplasmic retinoic acid binding protein (CRABP). It is then transported to the nuclear receptors, RXR and RAR resulting in transcription activity and gene regulation.

After the release of retinol, RBP loses its affinity for transthyretin forming apo-RBP. This gets altered by the kidney- reabsorbed and catabolised. Thus, one RBP carries only one retinol molecule before being degraded.

RBP4 GENE POLYMORPHISM[108]:

It has been shown that single nucleotide polymorphism (SNP) in the RBP4 promoter region is associated with 2 fold increase in risk of T2DM. The increase in RBP4 promoter activity is positively associated with its expression in the adipose tissue and also with BMI.

The SNP in the regulatory region of the RBP4 gene is identified as 803 GA polymorphism of rs3758539. This 803GA polymorphism is a functional variant that affects HNF1α binding, RBP4 transcription efficiency and plasma level of RBP4.

Dietary retinol is not associated with RBP4 polymorphism or risk of T2DM. RBP4 polymorphism is not associated with circulating levels of retinoids.

POLYMORPHISM OF RECEPTORS OF RBP4[107]:

STRA6 (Stimulated by retinoic acid gene homolog 6) is a cell surface receptor. It also acts as a cytokine receptor by activating JAK/STAT signal cascade by combining with RBP4.

STRA6 gene is located on chromosome 15q24.1 region. It has 20 exons and 19 introns. The association between STRA6 rs974456 and rs736118 polymorphism and T2DM in south Indian population has been established. They all are C/T dimorphic. The risk variants are CT heterozygous and TT homozygous.

Because of this SNP, that is change from C to T allele is due change of amino acid from methionine to tyrosine. This takes place in the c- terminal of STRA6, which is involved in signal transduction by phosphorylation. Tyrosine conversion has a potential impact in this signal cascading.

ROLE OF RBP4 IN PATHOGENESIS OF GESTATIONAL DIABETES MELLITUS:

INSULIN RESISTANCE IN SKELETAL MUSCLE[105,106]

Skeletal muscle is a major site of glucose disposal in the body. In GDM along with adipose tissue, it becomes insulin resistant. In the muscles, there is reduction of insulin stimulated uptake of glucose by GLUT4 receptors.

MECANISHM OF ACTION IN CAUSING
INSULIN RESISTANCE

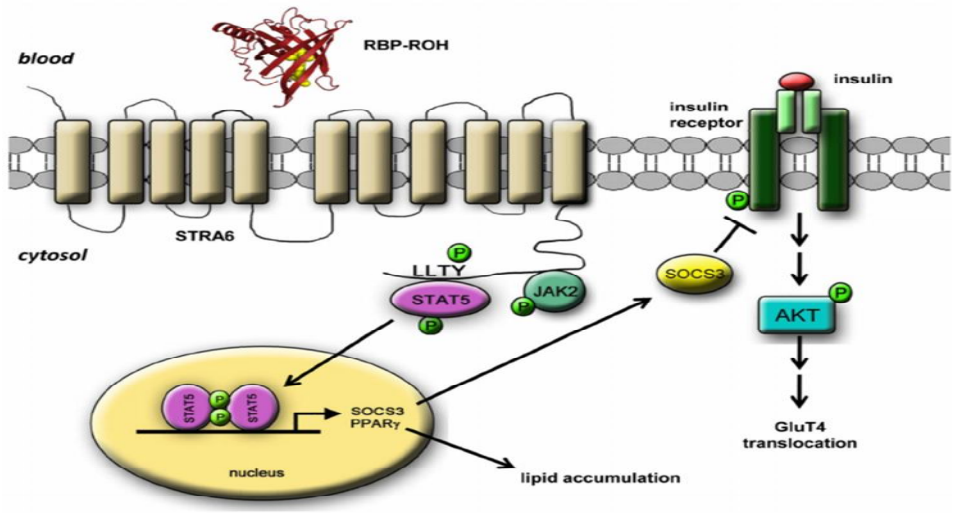

FIG 5: MECHANISM OF RBP4 IN CAUSING INSULIN RESISTANCE

IMAGE COURTESY: researchgate.net

Normally, the attachment of insulin to its receptor stimulates tyrosine phosphorylation of these receptors. In GDM, there is defective tyrosine phosphorylation due to instrinsic defect in the insulin signaling pathway. This is the major role of RBP4 in the pathogenesis of GDM. This is through stimulation of alternate JAK-STAT cascade.

- The cytosolic domain of STRA6 receptor of RBP4, contains a stretch of residues that conform to a consensus phosphotyrosine motif.
- Phosphotyrosines are often found on surface receptors, that transduces extracellular signals by activating JAK-STAT cascades.
- Thus the presence of phosphotyrosine motif in STRA6 suggests the possibility that, in addition to serving as vitamin A transporter, it may also

function as a signaling receptor which is activated by large amount of RBP4 in GDM.

- Thus RBP4 acts as an extracellular ligand that activates STRA6, which in turn modulates cellular response by triggering JAK/STAT signaling.
- The resultant action is abnormal phosphorylation of serine/threonine residues in the insulin receptor (IR) and insulin receptor substrate (IRS).

The intracellular substrate 1 (IRS 1) is a major docking protein in muscles. It regulates insulin uptake in insulin sensitive tissues. It has been shown that, the IRS-1 protein level is reduced by 30-50% in GDM. Also, there is increased 312-serine phosphorylation of these proteins, which inhibits PI-3 kinase activity.

Inhibition of PI-3 kinase activity, affects the phosphrylation of Akt, which is necessary for translocation of GLUT4 receptors.

Alternatively, increased serine phosphorylation of IRS-1 can be due to activation of *mTOR-p 70S6* pathway.

mTOR-p 70S6k1 and AMPK act as nutrient and energy sensors in the cellular level. AMPK is a negative regulator of mTOR. Its level is elevated in GDM.

RBP4 AND INSULIN RESISTANCE IN ADIPOSE TISSUE[109]:

"Obesity is a state of chronic low grade inflammation." The expansion of adipose tissue is characterized by increase in both adipocyte number and size. Many molecules secreted by adipocytes, promote adipose tissue inflammation.

Recent evidences suggest the possibility of pro-inflammatory pathway in RBP4-induced insulin resistance[102] in adipose tissue. The proportion of RBP4 that is apo-RBP4 is increased in obese persons, and also the ratio of RBP4 to retinol is increased in people with T2DM.

Similar to the action in the muscle, RBP4 can act through the JAK/STAT pathway through the RBPR2 receptor. The other way of action is mediated through stimulation of macrophages.

It has been shown that RBP4 acts independent of retinol to impair insulin signaling in adipocytes. It acts indirectly by inducing proinflammatory cytokine release from the macrophages. These actions by the Toll-like receptor 4(TLR4) cell surface receptor, involving the c-Jun N-terminal protein kinase (JNK) signaling pathway. The effect of RBP4 in inducing IL-6 synthesis is greater than in stimulating TNF.

"Peroxisome proliferator- activated receptor (PPAR) is a negative regulator of proinflammatory pathways in macrophages." The expression of PPAR was reduced by 50 to 70% by RBP4. Thus reduced PPAR expression is involved in the induction of proinflammatory state by RBP4.

Macrophages by acting through the adipocytes, play a critical role in the development of insulin resistance[103]. Insulin stimulates Akt phosphorylation, which is necessary for translocation of GLUT4 receptors.

RBP4 causes suppression of Akt phosphorylation in 3T3L1 adipocytes intermingled with macrophages, thus resulting in insulin resistance in adipose tissue. This shows that macrophage- derived proinflammatory cytokines induced by RBP4 mediated insulin resistance in adipose tissue.

This effect of RBP4 is mediated through the TLR4 and JNK pathway, independent of retinol binding to RBP4. Thus RBP4 is not the only adipose tissue derived factor, that is important for its inflammation and T2DM.

Others like leptin and FFA also induce proinflammatory cytokine production. But it has been **identified that RBP4 is a key endogenous protein that contributes to adipose tissue inflammation and insulin resistance by triggering interaction between innate and adaptive immune system.** These understanding of integration of inflammatory pathway and insulin resistance have therapeutic implications for T2DM.

RBP4 AND CORONARY ARTERY DISEASE[110]

Type II DM is characterized by inadequate insulin secretion as well as insulin deterioration and resistance. It is described as “silent disease”. T2DM is an independent risk factor for CAD. Risk factors including hyperglycemia, dyslipidemia, family history of the disease directly increases the mortality of CAD.

Insulin resistance acts as a strong linking factor between T2DM and CAD. It is characterized by malfunctioning adipocytes and greater amount of proinflammatory cytokines. There is

- low-grade elevation of acute phase reactants
- proinflammatory cytokine secretion and
- cell adhesion molecules.

The resulting subclinical inflammation causing insulin resistance correlates with pathogenesis of all phases of atherosclerosis.

- Persistent hyperinsulinemia causes raised levels of TGL, FFA and LDL as well as reduced HDL.
- Increased FFA activates innate immune system and there is resultant release of proinflammatory cytokines like TNF-α, IL6 etc.
- Cytokines mediate abnormal insulin signaling pathway in adipocytes and muscle causing insulin resistance.
- They disable the liver X receptors(LXRs) and increased accumulation of cholesterol.
- There is resultant release of inflammatory markers including CRP(C-Reactive protein), plasminogen inhibitor- 1 etc.
- Cytokines stimulate fibrinogen leading to CAD.
- Increased VLDL and FFA, causes characteristic diabetic dyslipidemia and increased plaque accumulation.

Increased Serum RBP4 level corresponds to the number of diseased arteries.

These striking features suggest a strong correlation between RBP4 and pathogenesis of CAD.

These findings are further supported by the higher RBP4 expression by the epicardial fat in coronary artery disease patients.

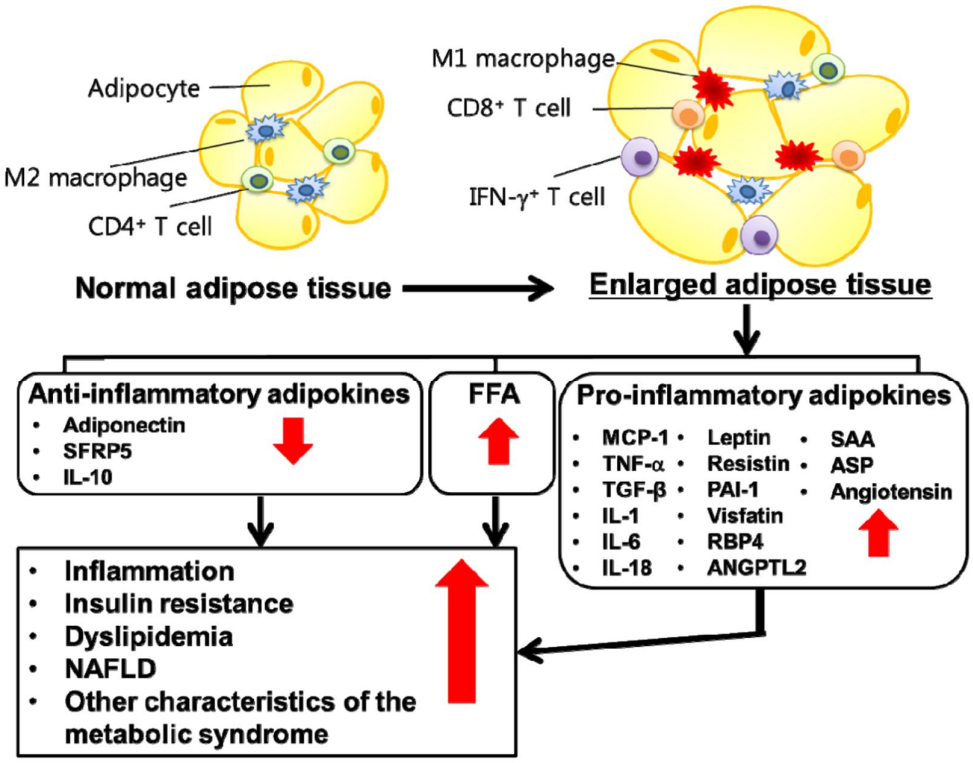

FIG 6: SECRETION OF INFLAMMTORY ADIPOKINES FROM ADIPOSE TISSUE

IMAGE COURTESY: International journal of molecular sciences

RBP4 IN METABOLIC SYNDROME:

Metabolic syndrome is a cluster of metabolic abnormalities, which includes

- Waist circumference(WC) >102 cm for men,>88 cm for women
- Triglycerides (TGs) >150 mg/dL
- High density lipoprotein (HDL) <40 mg/Dl for men and <50 mg/dL for women
- Blood pressure(BP) >130/85 mmHg
- FBS >100 mg/dL

It increases the risk of many chronic diseases like T2DM and CAD.

The prevalence of metabolic syndrome based on WHO criteria was 24% after 6 to 12 weeks postpartum in women with GDM. RBP4 level positively correlates with systolic hypertension, abdominal fat, fasting insulin concentration and insulin resistance.

RBP4 AND THYROID DISORDERS:[111]

Prevalence of subclinical hypothyroidism (SCH) is high in elderly population. Cardiac and metabolic risk factors are important in older individuals with abnormal thyroid function. Subclinical hypothyroidism (SCH) is an independent risk factor for CVD.

RBP4 level was found to be higher in SCH than in euthyroid state.It was significantly and positively correlated with TSH levels. Altered adipokine metabolism is related to metabolic disorders in SCH.

RBP4 in Non-alcoholic Fatty Liver Disease(NAFLD):[112]

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease. It is considered as the hepatic manifestation of metabolic syndrome. Insulin resistance (IR) is the pathophysiological basis of NAFLD. Non alcoholic steatohepatitis (NASH) is the most severe form of NAFLD resulting in fibrosis.

Significant inverse correlation was found between the stage of fibrosis and RBP4 levels. For each stage of increase in fibrosis, the mean RBP4 levels decreases by 3.06 mg/L.

- Serum RBP4 can be used as a potential marker to assess fibrosis progression in NAFLD.
- This noninvasive test also helps to distinguish NASH and simple steatosis.
- It helps to assess histological severity in patients with NAFLD.

The inverse relationship between RBP4 level and fibrosis is due to the fact that, retinoic acid is a supressor of type I collagen, expressed by the hepatic stellate cells. This is important in fibrinogenesis. Lower level of RBP4 is involved in activating stellate cells to overexpress and deposit type I collagen in liver.

Thus lower levels of RBP4 in patients with NAFLD can be used as a screening test to identify patients at a risk of developing NASH and advanced fibrosis.

RETINA DYSTROPHY, IRIS COLOBOMA, COMEDOGENIC ACNE SYNDROME:

There is deficiency of RBP4 levels in this syndrome. It is due to the mutations affecting the gene represented in its attachment to the target cells. Loss of functional RBP4 levels results in serum retinol deficiency. Reduced levels of retinol results in dry skin, increased susceptibility to infections and acne.

There is also degeneration of retina, ocular coloboma, impaired scotopic vision and loss of visual acquity.

Heterozygous mutations of Ile41Asn(rs121918584) and Gly75Asp (rs1218585) in RBP4 gene are associated with retinal degeneration.

MICROPHTHALMIA SYNDROMIC 9

Mutation of STRA6 receptors results in decreased uptake of retinol by the cells. This results in decreased photoreceptor formation within the cell. The defect results in various ocular abnormalities. It also causes various systemic effects in the cells which require retinoic acid for its differentiation and development.

STRA6 receptors are present in a variety of embryonic as well as adult cells and tissues. The mutations of STRA6 receptors can result in fatal Matthew-Wood syndrome.

This is characterized by

- multisystem malformations
- cardiac deformities and ocular defects.

LIPOPROTEINS AND TRANSPORTATION OF LIPID:[113]

Lipoproteins are micro-emulsions containing lipids and proteins. They are held together by non-covalent forces. Lipoproteins consists of a

- hydrophobic core made of mainly triglycerides and cholesteryl esters

- amphipathic coating composed of phospholipids, cholesterol and apoproteins.

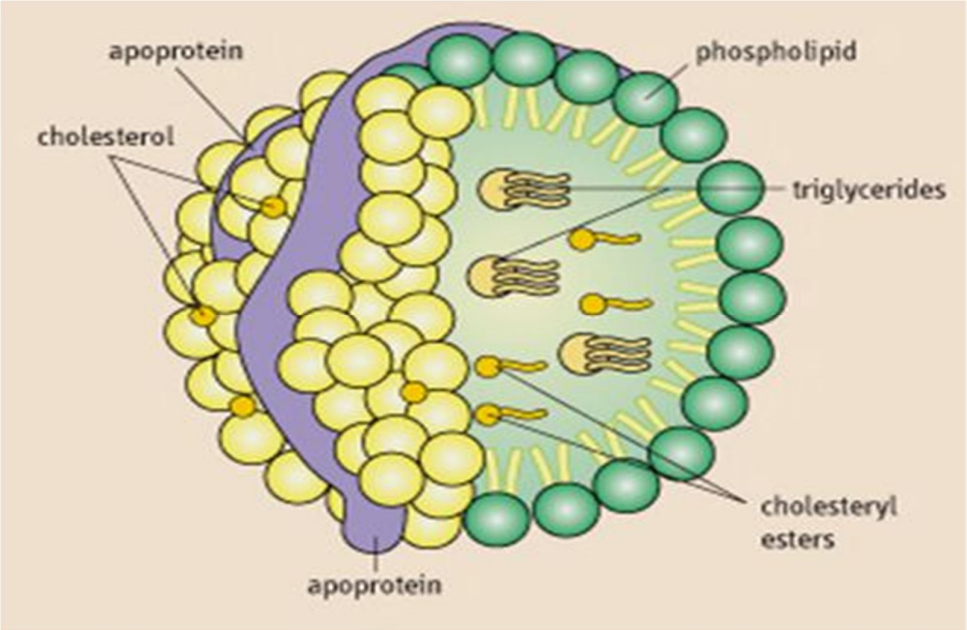

FIG 7: LIPOPROTEIN PARTICLE

IMAGE COURTESY: www.studentconsult.com

Lipoproteins differ in their lipid and protein composition. Based on their density, lipoproteins are classified as:

- Chylomicrons(<0.95g/mL)
- Very low density lipoproteins (0.95-1.006 g/mL)

- Intermediate density lipoprotein (1.006-1.019 g/mL)
- Low density lipoprotein (1.019-1.063 g/mL)
- High density lipoproteins (1.063-1.210g/mL)

CHYLOMICRONS:

- It is the largest lipoprotein
- It is formed in the intestine
- Major apoprotein in apo B-48
- It also contains apo A-I, A-II and A-IV
- It gets its apo C and apo E from HDL
- Mainly transports triglycerides of dietary origin

VERY LOW DENSITY LIPOPROTEINS

- Contains endogenously synthesized triglyceride.
- Transport TGL from liver to extra hepatic tissues.
- major apoprotein is apo B-100.
- It also contains apo C-I, apo C-II, apo C-III ,apo-E

INTERMEDIATE DENSITY LIPOPROTEIN

- Formed as an intermediate during conversion of VLDL to LDL.
- Its apoporotein copmposition is similar to VLDL.
- It transports TGL and cholesteryl esters.

LOW DENSITY LIPOPROTEIN

- Formed as the end product of VLDL metabolism

- Its major apoprotein is B-100
- Carries mainly cholesteryl esters.

HIGH DENSITY LIPOPROTEIN

- Smallest and most dense
- Nascent HDL is discoidal in shape. It accepts cholesterol and becomes spherical.
- Its apoproteins include apo A-I, apo A-II, apo A-IV, apo C-I, apo C-II.C-III and E.
- It is the donor of apo C and E to other lipoproteins.
- Apo C is a co-factor for lipoprotein lipase enzyme and apo-E is required for hepatic uptake of lipoproteins.

CHYLOMICRONS AND VLDL METABOLISM:

Lipoprotein lipase enzyme is found attached to the endothelium of capillaries by negatively charged heparin sulphate. It plays a major role in TGL metabolism.

The Lipoprotein lipase isoform in the heart has a low Km than the isoform in the adipose tissue. This allows the heart to make a better use of TGL during starvation. The enzyme requires cofactors like phospholipids and apo C-II. Its action is inhibited by apo C-III and apo A-II. Triglycerides are progressively hydrolysed to diacylglycerol, mono-acylglycerol and finally fatty acid and glycerol. The released FFA is bound to albumin and transported to tissues.

Insulin enhances lipoprotein lipase synthesis and its translocation to the luminal surface of capillaries. The action of lipoprotein lipase is to decrease the lipid content of chylomicrons and VLDL, which results in the formation of chylomicron remnant and VLDL remnant. They contain cholesterol and cholesteryl esters. VLDL remnant is called IDL.

Chylomicron remnant is taken up by the liver through receptor mediated endocytosis. The cholesteryl esters and left over TGL are metabolized by hepatic lipase. This uptake is enhanced by apo-E by 2 apo-E dependent receptors

(1) LDL (apo B-100, apo E) receptor

(2) LDL receptor related protein (LRP)

IDL can be taken up by liver via LDL receptor or it can be converted into LDL. LDL mainly contains cholesterol and cholesteryl esters.

LDL METABOLISM:

LDL is metabolized in liver as well as extrahepatic tissues. The LDL receptors are specific for apo B-100, not for B-48. The carboxy terminal of apo B-100 acts as a ligand for LDL receptor. It also takes up lipoproteins rich in apo- E. After binding to the receptor, LDL is endocytosed. The endocytosed vescicles fuses with lysosome to form endosome with the receptor. A strong positive correlation has been established between blood levels of LDL and atherosclerosis. A defect in LDL receptor mediated uptake results in familial hypercholesterolemia.

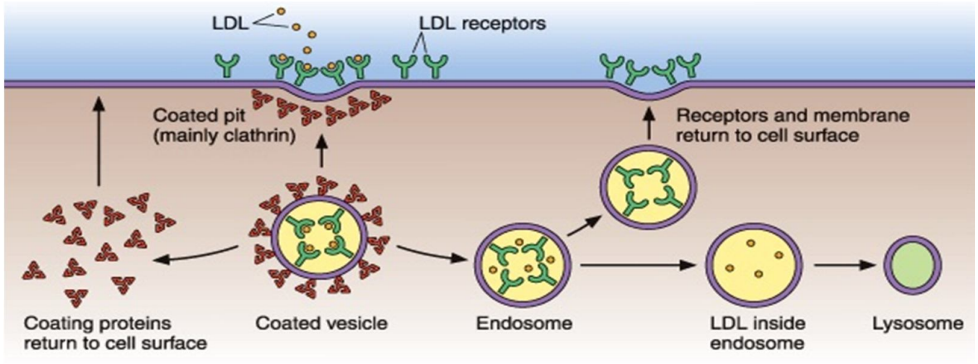

Fig 8: LDL RECEPTOR MEDIATED ENDOCYTOSIS

IMAGE COURTESY: McGrawhill. companies.Inc

HDL METABOLISM:

HDL is synthesized and secreted by liver and intestine. Apo-C and apo-E are synthesized in the liver. They are secreted along with HDL from which it is transferred to other lipoproteins.

Nascent HDL (pre-β HDL) is discoidal in shape. It has a phospholipid bilayer with apo-A and cholesterol. Apo-A1 acts as an activator of LCAT enzyme. LCAT binds to nascent HDL and converts cholesterol to cholesteryl ester by transfer of acyl group from the membrane phospholipid. Lysolecithin is formed as a by-product.

As HDL becomes loaded with cholesteryl ester, it acquires a spherical shape converting into HDL3 and finally HDL2. HDL2 is taken up by class B scavenger receptor B1 (SR-B1) present in the cells. In liver and steroidogenic tissues, SR-B1 binds to HDL through apo A-I and cholesteryl ester is delivered into the cell.

In other tissues, the receptor mediates efflux of cholesterol from the tissues and its acceptance by HDL. Then HDL transports cholesterol to the liver where it is excreted in bile. This is called reverse cholesterol transport.

HDL3 accepts cholesterol from peripheral tissues and gets esterified by LCAT to form cholesteryl ester. This causes increase in size of HDL, which is now called the HDL2. This delivers cholesteryl ester to liver through the SR-B1or undergoes hydrolysis of HDL by hepatic lipase and endothelial lipase reforming HDL3. The interchange between HDL2 and HDL3 is called HDL cycle.

Another mechanism of reverse cholesterol transport occurs through the ABCA1 and G1 transporters. These transporters, couple the hydrolysis of ATP to binding of substrate, and facilitates its transport across membrane. ABCG1 transports cholesterol from cells to HDL. ABCA1transports cholesterol from cell to pre-β HDL or apo A-I containing nascent HDL. Pre-β form of HDL is the most potent form of HDL facilitating cholesterol efflux from the cells.

Plasma HDL level usually varies reciprocally with TGL levels and directly with lipoprotein lipase activity. Also HDL concentration is inversely related to atherosclerosis.

FREE FATTY ACID METABOLISM:

These are otherwise called as non esterified fatty acids. They are formed as a result of breakdown of TGL in adipose tissue by the action of lipoprotein lipase. They are transported in the plasma bound to albumin. They are either rapidly

oxidized in the mitochondria or esterified to form TGL. The FFA uptake by the cells is directly proportional to its concentration in the plasma, that is, it indicates the rate of lipolysis.

On reaching the plasma membrane, FFA dissociates from albumin and transported into cell by membrane fatty acid transport protein. It is a cotransporter of Na^+. Inside the cytosol it is bound by intracellular fatty acid binding protein.

REGULATION OF VLDL SYNTHESIS:

Hepatic triglyceride synthesis acts as a stimulator for VLDL synthesis. Fatty acids essential for TGL synthesis is derived from

1. From acetyl CoA, in well fed state
2. High fat diet
3. High insulin level and low glucagon
4. Ethanol intake
5. Enhanced lipolysis

Triglyceride is normally not stored in the liver. Instead, it is packed into VLDL and secreted immediately. VLDL is synthesized in endoplasmic reticulum by the enzyme microsomal triacylglycerol transfer protein. The enzyme transfers TGL from cytosol to endoplasmic reticulum, where it is packed with cholesterol, apo B-100 and phospholipids. Imbalance in the synthesis and secretion of TGL causes fatty liver.

TRIGLYCERIDE STORAGE:

Triacylglycerol is stored in the adipose tissue as lipid droplets. It is continuously hydrolysed and re-esterified. The balance between these two cycles determines the circulating non-esterified FFA level in the blood.

REGULATION OF ESTERIFICATION OF FATTY ACID:

Acyl CoA and glycerol-3-phosphate combine to form triacylglycerol. The enzyme glycerol kinase is absent in adipose tissue. So, adipose tissue cannot use glycerol to synthesize TGL.

Glycerol-3-phophate is obtained from glycolysis. Dihydroxyacetone phosphate, an intermediate in glycolysis, is reduced to glycerol-3-phosphate. This requires NADH. Thus, the availability of glycerol-3-phosphate regulates synthesis of TGL.

REGULATION OF LIPOLYSIS:

TGL is hydrolyzed to FFA and glycerol by hormone sensitive lipase in adipose tissue. As glycerol cannot be utilized by adipose tissue, it is transported to the liver and kidney for further phosphorylation and utilization.

The FFA is converted to acyl CoA by acyl CoA synthetase and re-esterified with glycerol-3-phosphate to form TGL.

Thus there is a continuous cycle of lipolysis and re-esterification .If there is excess lipolysis, the non-esterified free fatty acid (NEFA) binds with albumin and

circulates. When there is excess of glycolysis, much of glycerol-3-phosphate is available for esterification, promoting TGL synthesis. Increased utilization of glucose prevents efflux of FFA from adipose tissue.

REGULATION BY INSULIN:

Adipose tissue is one of the major site of action of insulin.

- Insulin inhibits lipolysis and release of FFA
- Promotes uptake of glucose via GLUT4 receptors
- Enhances the utilization of glucose as substrate for fatty acid synthesis
- Promotes TGL synthesis.
- Inhibits synthesis of cAMP at adenylyl cyclase level.
- Stimulates phophodiesterase by decreasing cAMP concentration.
- Enhances protein phosphatase ,dephosphorylates hormone sensitive lipase, making it inactive.
- It increases the activity of the following enzymes,which enhance fatty acid and TGL synthesis.

1. Pyruvate dehydrogenase
2. Acetyl CoA carboxylase
3. Glycerol phosphate acyl transferase

GLYCATED HEMOGLOBIN[114,115]

"Glycation is the nonnzymatic addition of a sugar residue to amino groups of proteins". Adult hemoglobin is made of four polypeptide chain (2α,2β). It usually consists of- 97% of HbA, 2.5% of HbA_2 and 0.5% of HbF.

Glycosylation of specific proteins occur under precise enzymatic control which makes the protein functional, such as those proteins involved in integrity of plasma membrane and secretory proteins. On the other hand, in conditions of high level of blood sugar levels, there is non-physiological 'browning' reactions of proteins. But formation of HbA_1c is physiological and non-enzymatic.

Chromatographic separation shows several minor types, which includes- HbA_1a, HbA_1b, HbA_1c which are collectively known as HbA_1. They are also known as fast hemoglobin, glycohemoglobis or glycated hemoglobin. IUPAC recommends the use of the term neoglycoprotein.

HbA_1c is formed by the condensation of glucose with the N-terminal valine residue of both the β chain HbA.This first forms an unstable Schiff base. Later the Schiff base dissociates or undergoes an amadori rearrangement thus forming a stable ketoamine HbA_1c. It forms 80% of HbA_1.

Others,

- HbA_1a1 has fructose 1,6-diphosphate
- HbA_1a2 has glucose-6-phosphate
- HbA_1b has pyruvic acid

Formation of glycated hemoglobin is an irreversible process. Its concentration in blood depends on (1) life span of RBCs(120days)

Blood Glucose Level

CLINICAL SIGNIFICANCE:

Alteration in tissue structure and function due to hyperglycemia is one of the pathogenic mechanisms resulting in complications of diabetes mellitus.

Of the many pathogenic mechanisms due to hyperglycemia, alteration in tissue structure and function is one involved in complications of diabetes. Changes in the matrix components by glycation causes alteration in cell behavior. These changes includes

- changes in cell spread
- phosphorylation of several intracellular signaling molecules
- expression of extensively glycated extracellular matrix.

The glycation also affects activity of enzymes like glutathione S-transferase, Cu-Zn superoxide dismutase. This also further contributes to the pathogenesis of diabetes.

The changes in the structure of the tissue as well as its function are slow but cumulative. This gives a long lag time between the disease onset and progress to complications.

Rate of formation of glycated Hb is directly proportional to the blood glucose levels, hence glycated Hb concentration represent an integrated value for assessing glucose over the previous 8 to 12 weeks. Using such values has an advantage of not having day-to-day fluctuations nor it's value gets affected by recent food intake or exercise.

As the interpretation of values depends on the lifespan of RBCs it is difficult to infer the results directly in patients with hemolytic disorder or with recent history of severe blood loss, so it is compared with the previous results.

In the year 2009, HbA1c was recommended by the International Expert committee as a diagnostic test for diabetes with a threshold of >6.5%. The diagnostic test should be standardized to DCCT(Diabetes Control and Complication Trial) reference assay or method certified by "National Glucohaemoglobin Standardisation Programme"(NGSP).

ADVANTAGES OF USING HbA1c:

1) As a test for glycemia- less intraindividual variation.
2) Better predictor of complications like CAD than FBS or OGTT.
3) Used for monitoring of glucose in diabetic patients.

Aims & Objectives

AIM

1. To estimate the level of serum retinol binding protein 4 (RBP4) in women with gestational diabetes mellitus.
2. To estimate the lipid profile in patients with gestational mellitus.

OBJECTIVES

1. To correlate the level of serum retinol binding protein protein 4 (RBP4) and lipid profile in patients with gestational diabetes mellitus.
2. To correlate the level of serum retinol binding protein 4 (RBP4) and HbA_1c in patients with gestational diabetes mellitus.

Materials & Methods

MATERIALS AND METHODS

This is a case control study and the study protocol was approved by the Institutional Ethics Committee of Madras Medical College, Chennai and a copy of it has been enclosed. Madras.

STUDY PERIOD: October 2016 to June 2017

SUBJECT SELECTION:

90 pregnant women attending the diabetology OP were selected for the study. They were divided in to 60 cases and 30 controls based on the OGTT report. Of the cases, it was further subgrouped as 30 primiparous women and 30 multiparous women.

INCLUSION CRITERIA:

GROUP I : Primi with gestational diabetes mellitus of age 21 to 40 years

GROUP II : Multi with gestational diabetes mellitus of age 21 to 40 years.

GROUP III : Age and parity matched healthy pregnant women.

EXCLUSION CRITERIA:

- Women with pre-existing diabetes or hypertension.
- Patients with liver disease.
- Patients with acute illness; infection.
- Known case of cardiovascular disease.
- Smoking, alcoholism.
- Patients with history of fetal anomalies.
- Other endocrinological disorders.

SAMPLE COLLECTION:

6 mL of blood sample was collected from all subjects after overnight fasting. 3mL of was transferred to serum tube and 3 mL was transferred to K_2-EDTA tube. After adequate clotting, the serum sample was centrifuged and serum was aliquoted. The following investigations were done.

Tube	Anticoagulant	Volume of blood	Investigations
Tube 1	EDTA	3mL	FASTING BLOOD GLUCOSE, HbA1c, FASTING LIPID PROFILE
Tube 2	-	3 mL	Sr.RBP4

The aliquoted serum for RBP4 was stored immediately at - 20^{o}C.

BIOCHEMICAL INVESTIGATIONS

ESTIMATION OF SERUM RETINOL BINDING PROTEIN 4:

METHOD:

Enzyme linked immunosorbent assay–non-competitive sandwich.

PRINCINPLE:

RBP4 in the standard and the test sample is added to the wells which are pre-coated with RBP4 monoclonal antibody. Then, a biotin conjugated anti-human RBP4 antibody is added and it binds to human RBP4. After incubation, the unbound biotin conjugated anti-human RBP4 antibody is washed away. Streptavidin-HRP is added. This binds to the biotin conjugated anti-human RBP4

antibody. Unbound streptavidin-HRP is washed off. Then, substrate solution is added.

The intensity of color developed is proportional to the amount of human RBP4. The reaction is terminated by adding an acidic stop solution. The absorbance is measured at 450nm n an automated microplate reader.

REAGENTS:

1. Pre-coated ELISA plate.
2. Standard solution.
3. Standard diluents.
4. Streptavidin-HRP.
5. Stop solution.
6. Substrate solution A.
7. Substrate solution B.
8. Concentrated wash buffer.
9. Biotin conjugated anti-human RBP4 antibody.

REAGENT PREPARATION:

1. All the reagents should be brought to room temperature before use.
2. Standard preparation: A standard stock solution of concentration 192mg/L is taken. It is then diluted successively as shown to make the standards of other concentrations.

192mg/L 96mg/L 48 mg/L 24mg/L 12mg/L 6mg/L

96mg/L	Standard No.5	120µl of Original standard + 120 µl Standard diluent
48mg/L	Standard No.4	120µl of Standard No.5 + 120µl Standard diluent
24mg/L	Standard No.3	120µl of Standard No.4 + 120µl Standard diluent
12mg/L	Standard No.2	120µl of Standard No.3 + 120µl of Standard diluent
6mg/L	Standard No.1	120µl of Standard No.2 + 120µl of Standard diluent

PROCEDURE:

1. 50µl of standard was added to the standard wells.
2. 40µl of sample was added to the sample wells and then anti-RBP4 antibody was also added to the sample wells.
3. 50µl of streptavidin-HRP was added to the sample as well as the standard wells. But not to the blank well.
4. The contents were mixed well and covered with a plate sealer.
5. It was incubated for 60 minutes at 37^{o}C.
6. After removing the sealer, the plate was washed for 5 times with wash buffer. All the wells were aspirated and washed 5 times with wash buffer.
7. The plate was blotted onto paper towels.
8. 50µl of substrate solution A and then 50µl of substrate solution B were added to all the wells.

9. Incubated for 10 minutes at 37^{o}C.
10. 50µl of stop solution was added to each well.
11. The blue color changed to yellow color immediately.
12. The OD(optical density) of each well was determined using microplate reader at 450nm within 30 minutes of adding the stop solution.

STANDARD CURVE RANGE: The kit has working range from 0.5mg/L to 180mg/L.

A standard curve was constructed by plotting the average OD for each standard in the Y-axis and the concentration in the X-axis and a best fit curve was drawn through the points on the graph.

SENSITIVITY: 0.26mg/L

Estimation of PLASMA GUCOSE:

Method:

Glucose Oxidase-Peroxidase method.

Kit used: System pack

Principle:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Colored quinoneimine} + 2H_2O$$

The intensity of the red colored quinineimine complex formed is directly proportional to concentration of glucose in the sample. Absorbance is measured spectrophotometrically at 505nm.

Reagent composition:

ACTIVE INGREDIENTS	CONCENTRATION
Phosphate buffer	250mmol/L
Glucose oxidase	>25U/MI
Peroxidase	>2U/mL
4-aminoantipyrine	0.5 mmol/L
Phenol	5 mmol/L

The kit was calibrated using XSYS0034 XL MULTICAL CALIBRATOR

The glucose concentration of the XLMULTICAL is 197.7mg/dL

Assay parameters:

Mode	End point
Wavelength 1 (nm)	505
Wavelength 2 (nm)	670
Sample volume (µL)	5/10
Reagent volume (µL)	500/1000
Incubation time (min)	5
Incubation temperature (^{o}C)	37
Normal low (mg/dL)	74
Normal high (mg/dL)	100
Linearity low (mg/dL)	2.34
Linearity high (mg/dL)	450
Blank with	Reagent
Absorbance limit	0.2
Unit	mg/dL

Reference Interval:

Fasting blood glucose 74-100 mg/dL

LIPID PROFILE:

SERUM TOTAL CHOLESTEROL

Method:

CHOD-PAP METHOD (Cholesterol oxidase-phonol-aminophenazone peroxidase)

Principle:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{CHE}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + 4\text{AAP} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinineimine dye} + 4H_2O$$

The intensity of the red colored complex formed is directly proportional to concentration of cholesterol. The absorbance is measured at 505nm.

Reagent Composition:

ACTIVE INGREDIENTS	CONCENTRATION
Good's Buffer (pH– 6.4)	50mmol/L
Phenol	5mmol/L
4-aminoantipyrine	0.3 mmol/L
Cholesterol esterase	>200 U/L
Cholesterol oxidase	>50U/L
Peroxidase	>3kU/L

The kit was calibrated using XSYS0034 XL MULTICAL calibrator .The cholesterol concentration of the calibrator is 164.5 mg/Dl

Assay Parameters:

MODE	End point
Wavelength 1 (nm)	505
Wavelength 2 (nm)	670
Sample volume (µL)	5/10
Reagent volume (µL)	500/1000
Incubation time (min)	5
Incubation temperature (^{o}C)	37
Normal low (mg/dL)	0
Normal high (mg/dL)	100
Linearity low (mg/dL)	4.2
Linearity high (mg/dL)	695
Blank with	Reagent
Absorbance limit(max)	0.2
Units	mg/dL

Reference Interval:

Desirable blood cholesterol <200 mg/dL

Borderline high blood cholesterol 200-239 mg/dL

High blood cholesterol >239 mg/dL

SERUM TRIGLYCERIDES

Method:

GPO-PAP method (glycerophosphate oxidase- phenol- aminophenazone peroxidase)

Principle:

$$\text{Triglycerides} + H_2O \xrightarrow{\text{LPL}} \text{Glycerol} + \text{Free Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK, } Mg^{2+}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4AAP} + \text{4-Chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + H_2O$$

The intensity of the red colored compound formed is directly proportional to concentration of triglyceride present in the sample. The absorbance is measured at 505 nm.

Reagent Composition:

ACTIVE INGREDIENT	CONCENTRATION
Good's buffer (pH 7.2)	50mmol/L
ATP	2mmol/L
Mg^{2+}	15mmol/L
4-amonoantipyrine	0.5 mmol/L
4-chloroPhenol	4 mmol/L
Glycerokinase	>0.4kU/L
Glycerol-3-phosphate oxidase	>0.5 kU/L
Peroxidase	>2 kU/L
Lipoprotein lipase	>2 kU/L

The kit was calibrated using XSYS0034 XL MULTICAL calibrator . The concentration of triglyceride in the calibrator is 137.6 mg/dL

Assay Parameters:

Mode	END POINT
Wavelength 1 (nm)	505
Wavelength 2 (nm)	700
Sample volume (µL)	5/10
Reagent volume (µL)	500/1000
Incubation time (min)	5
Incubation temperature (^{o}C)	37
Normal low (mg/dL)	0
Normal high (mg/dL)	150
Linearity low (mg/dL)	9.7
Linearity high (mg/dL)	1062
Blanking with	Reagent
Absorbance limit (max)	0.5
Units	mg/dL

Reference interval:

Normal <150 mg/dL

High 150-199 mg/dL

Hypertriglyceridemia 200-499 mg/dL

Very high >499 mg/dL

HDL CHOLESTEROL

METHOD:

Phosphotungstic acid method

Principle:

$$\text{Serum/plasma} \xrightarrow{\text{Phosphotungstate, } Mg^{2+}} \text{HDL in supernatant + precipitate}$$

The precipitate contains chylomicrons, VLDL & LDL. The HDL obtained from the above step is estimated using Erba cholesterol reagent.

Precipitating Reagent:

Phosphotungstic acid	0.77 mmol/L
Magnesium chloride	17.46 mmol/L

The kit was calibrated using HDL standard of concentration of 25 mg/dL

Assay Parameters:

Mode	End point
Wavelength 1 (nm)	505
Wavelength 2 (nm)	670
Sample volume (µL)	25/50
Reagent Volume (µL)	500/1000
Incubation time (min)	5
Incubation temperature (^{o}C)	37
Normal low (mg/dL)	42
Normal high (mg/dL)	79.5
Linearity low (mg/dL)	0
Linearity high (mg/dL)	125
Blank with	Reagent
Absorbance limit (max)	0.2
Unit	mg/dL

Reference interval:

Adult male : 35-55 mg/dL

Adult female : 45-65 mg/dL

LDL CHOLESTEROL

It is calculated using Friedwald formula. It is given as:

LDL Cholesterol = TC-HDL-(Triglycerids/5) mg/dL

The above formula holds for triglycerides value less than 400 mg/dL .

Reference Interval:

Based on risk of coronary heart disease:

Optimal	-	< 100 mg/dL
Near optimal	-	100 to 129 mg/dL
Borderline high	-	130 to 159 mg/dL
High	-	160 to 189 mg/dL
Very high	-	>189 mg/dL

ESTIMATION OF HbA1C:

METHOD:

Particle enhanced immunoturbidimetric method. HbA1C is measured directly without measuring total Hemoglobin.

PRINCIPLE:

Total hemoglobin and HbA_1c present in the hemolysed blood, bind with same affinity to particles in R1. The amount of binding is proportional to the relative concentration of both the substances in blood. Mouse anti-human HbA_1c monoclonal antibody (R2a) binds to the particle bound HbA_1c. Goat anti-mouse IgG polyclonal antibody (R2b) interacts with the mouse anti-human HbA_1c antibody and agglutination takes place. The absorbance is proportional to the

HbA_1c bound to particles, which in turn is proportional to the percentage of HbA_1c in the sample.

REAGENT COMPOSITION:

R1	Buffer	20 mmol/L
	Latex	1.5%
R2a	Buffer	10 mmol/L
	Mouse anti-humanHbA1c monoclonal antibody	5.5 mg/dL
R2b	Buffer	1 mmol/L
	Goat anti-mouse IgG polyclonal antibody	67 mg/dL
R3	Hemolysing solution	

R2a:R2b is 2:1

ASSAY PARAMETERS

Wavelength	660nm
Temperature	37^oC
Sample volume	30µl
Reagent 1	1000 µl
Incubation time	5 mins
Reagent 2a/2b	500 µl
Incubation time	5 mins

LINEARITY: 3.98 % to 15.42 % according to NGSP

Statistical Analysis

STATISTICAL ANALYSIS

Statistics:

- Data was analysed using SPSS software version 16.0 and P value less than 0.05 was considered statistically significant. Continuous variables were presented as mean ± SD (standard deviation) and categorical variables were represented as frequencies and percentages.
- Height, weight, BMI, FBS, HbA_1c, RBP4, T.Cholesterol, triglyceride, HDL and LDL were compared between the study groups by Student t-test.
- Comparison between primi with mutiparous and control group was done by One way ANOVA.
- Family history and previous history were analysed by Chi square test.
- Correlation of parameters namely RBP4 with triglycerides and HbA1c were found out by Pearson correlation analysis.
- Stepwise linear regression analysis was performed to evaluate the mathematical relationship between RBP4 and other variables in patients with GDM.

Results

MASTER CHART - CONTROL

S. NO.	AGE	HEIGHT (mts)	WEIGHT	BMI	FBS	HbA1c	RBP4	T.CHOL	TGL	HDL	Hb%	Family h/o	Previous h/o
1	22	1.49	58	26.12	55	4.6	20.75	251	237	46	9.8	NO	
2	22	1.46	60	28.15	70	4.7	17.68	280	367	72	10.5	NO	
3	29	1.52	72	31.16	62	6.2	31.25	155	234	27	12.1	NO	
4	34	1.6	70	27.34	82	6.6	34.72	156	235	56	10	NO	
5	24	1.49	59	26.58	89	5	32.36	191	282	52	11.2	NO	
6	20	1.52	53	22.94	83	4.2	27.76	220	135	87	11.4	YES	
7	22	1.56	62	25.48	74	4.4	15.49	208	228	44	12.5	NO	
8	24	1.53	50	21.36	93	4.9	24.73	197	247	42	10.5	NO	
9	32	1.6	76	29.69	47	6.3	18.8	204	280	58	10.6	NO	
10	27	1.49	52	23.42	77	4.8	29.5	259	229	63	9.8	NO	
11	19	1.51	56	24.56	65	4.8	25	245	247	38	10.6	NO	
12	36	1.48	51	23.28	78	4.5	30	145	137	44	10.3	YES	
13	26	1.57	63	25.56	104	5.4	25.7	162	233	50	10	NO	
14	38	1.5	75	33.33	82	4.9	19.84	222	128	40	10.1	YES	NO
15	30	1.5	38	16.89	50	5.6	28.05	164	148	34	12	NO	NO
16	20	1.5	48	21.33	65	5.5	17.39	194	76	33	13	NO	NO
17	26	1.4	66	33.67	80	5	19.75	238	246	37	12.2	NO	NO
18	27	1.54	78	32.89	66	5.2	18.93	286	208	56	13	NO	NO
19	31	1.64	83	30.86	86	4.9	20.11	189	323	61	11.6	NO	NO
20	23	1.56	48	19.72	70	4.8	23.45	243	186	48	10.4	NO	NO
21	24	1.48	55	25.11	74	5.1	27.56	242	267	70	10.6	NO	NO
22	35	1.64	54	20.08	96	5	29	244	246	49	12	NO	NO
23	32	1.48	74	33.78	70	5.1	15.6	195	228	29	11	NO	NO
24	27	1.52	51	22.07	89	5.3	24.48	172	177	42	13	NO	NO
25	29	1.49	54	24.32	66	5.2	11.4	223	260	59	11.6	YES	NO
26	32	1.52	65	28.13	94	4.9	15.32	224	189	38	11.4	NO	NO
27	28.00	1.49	54.00	24.32	78.00	5.10	17.30	221.00	176.00	44.00	12.00	NO	NO
28	31	1.5	63	28.00	74	5.2	20.4	170	189	46	10.8	NO	NO
29	30	1.4	65	33.16	77	4.9	18.9	190	221	44	11	YES	NO
30	29	1.42	66	32.73	79	5.2	20.1	164	167	33	12	NO	NO

MASTER CHART - CASES PRIMIPAROUS

S. NO.	AGE	HEIGHT (mts)	WEIGHT	BMI	FBS	HbA1c	RBP4	T.CHOL	TGL	HDL	Hb%	Family h/o	Previous h/o
1	26	1.53	67	28.62	98	6.1	71.87	213	396	48	12.1	NO	
2	23	1.55	72	29.97	101	6.3	43.96	209	292	53	11	YES	
3	28	1.49	66	29.73	114	5.9	49.41	219	226	56	10.2	YES	
4	29	1.49	75	33.78	97	5.8	48.18	220	248	39	12.2	NO	
5	22	1.48	64	29.22	99	5.9	56.26	225	228	52	9.6	NO	
6	23	1.52	103	44.58	104	5.6	48.18	200	231	51	10.8	NO	
7	24	1.47	65	30.08	98	5.8	53.5	201	278	56	10	YES	
8	23	1.58	84	33.65	114	6.3	59.5	264	254	38	11.2	YES	
9	22	1.52	52	22.51	81	8.9	67.3	228	220	46	10.6	NO	
10	25	1.54	92	38.79	102	6.8	82.8	283	279	56	10.8	NO	
11	23	1.48	81	36.98	198	10.1	43.1	261	238	36	12.1	YES	
12	20	1.47	44	20.36	140	7.2	51.11	220	225	33	9.8	NO	
13	30	1.52	74	32.03	97	5.4	45.12	253	293	47	10	YES	
14	26	1.55	68	28.30	104	5.8	49.6	218	233	36	12.4	NO	
15	26	1.49	47	21.17	127	6.4	44.6	251	264	60	9.9	YES	
16	29	1.54	76	32.05	96	6	91	280	306	38	12.1	NO	
17	25	1.5	81	36.00	98	6.8	56.8	209	279	38	10.4	YES	
18	33	1.46	73	34.25	94	6.1	33.8	181	176	54	11.2	YES	
19	30	1.5	63	28.00	108	6.8	51.9	228	220	55	10.2	NO	
20	29	1.48	75	34.24	112	6.2	51.7	90	267	37	10.6	YES	
21	29	1.43	50	24.45	99	5.9	85	186	243	41	11.2	NO	
22	26	1.58	71	28.44	111	5.8	38.5	203	207	51	12.2	NO	
23	26	1.5	66	29.33	97	5.7	45.32	224	224	35	11.2	NO	
24	22	1.52	47	20.34	176	7.1	53.9	204	268	36	11.9	YES	
25	33	1.46	73	34.25	84	5.2	43.4	191	198	52	11.3	YES	
26	23	1.53	40	17.09	116	5.7	46.8	226	258	48	10.2	NO	
27	24	1.54	61	25.72	106	5.9	47.24	221	228	43	11.4	NO	
28	22	1.48	60	27.39	92	6.3	46.44	222	360	53	10.5	NO	
29	23	1.48	46.00	21.00	102.00	6.20	44.14	207.00	#####	50.00	10.20	NO	
30	26	1.5	54		96	5.4	43.6	212	287	38	11	YES	

MASTER CHART - CASES MULTIPAROUS

S. NO.	AGE	HEIGHT (mts)	WEIGHT	BMI	FBS	HbA1c	RBP4	T.CHOL	TGL	HDL	Hb%	Family h/o	Previous h/o
1	33	1.49	70	31.53	101	6.8	48.08	204	220	34	9.6	NO	NO
2	25	1.5	56	24.89	96	6.7	42.6	213	308	36	11.6	NO	NO
3	27	1.5	73	32.44	102	6.9	47.4	259	236	53	10.2	YES	NO
4	2	1.48	75	34.24	106	6.8	39.6	205	217	37	11.2	YES	NO
5	42	1.52	53	22.94	99	6.6	47.5	206	270	50	10.8	NO	YES
6	29	1.59	70	27.69	106	7	36.9	235	214	44	11.4	NO	NO
7	40	1.65	79	29.02	102	6.3	49.9	302	281	50	10.2	YES	NO
8	27	1.5	64	28.44	100	6.9	45.13	227	278	54	10.8	YES	NO
9	27	1.51	79	34.65	115	8	43.18	191	223	40	11.2	NO	NO
10	27	1.5	61	27.11	107	6	47.54	239	248	50	10.8	NO	YES
11	26	1.56	69	28.35	106	5.9	46.58	202	237	27	11.4	NO	YES
12	36	1.52	55	23.81	96	6.9	48.89	208	349	37	10.6	YES	YES
13	28	1.49	88	39.64	104	7.6	42.29	205	294	31	12.1	NO	NO
14	29	1.5	86	38.22	109	6.6	55.13	240	282	33	10.2	NO	NO
15	38	1.54	80	33.73	108	6.2	46.95	244	305	44	9.5	YES	YES
16	35	1.5	80	35.56	106	5.7	40.55	358	336	58	9.6	YES	YES
17	31	1.47	55	25.45	129	6.8	38.22	206	249	54	10	NO	YES
18	30	1.54	74	31.20	104	6.3	45.57	204	191	50	10.4	YES	YES
19	33	1.52	64	27.70	108	8.1	93	238	285	48	10.7	YES	NO
20	36	1.49	72	32.43	109	6.2	46.23	235	306	35	11.2	NO	NO
21	24	1.6	73	28.52	108	6.9	42.46	235	305	54	10.2	YES	NO
22	22	1.5	52	23.11	106	8.7	46.67	262	268	56	10.4	NO	NO
23	26	1.51	84	36.84	143	6.6	38.55	208	362	48	11.6	NO	NO
24	30	1.52	76	32.89	134	7.2	37.18	254	295	59	10.4	NO	NO
25	27	1.56	90	36.98	123	7.9	48.98	246	229	58	11.2	YES	NO
26	27	1.49	64	28.83	102	5.9	45.68	238	260	52	10.6	NO	NO
27	30	1.49	67	30.18	95	6.4	44.8	254	230	53	10.9	NO	NO
28	26	1.48	68	31.04	93	5.6	74.84	239	245	35	10.2	NO	YES
29	28	1.47	64	29.62	98	5.8	51.65	245	265	30	10.2	NO	NO
30	29	1.56	75	30.82	91	6.3	57.46	223	253	53	10.5	YES	NO

RESULTS AND STATISTICS

TABLE-1 : Age distribution of study Subjects

Age Group (in Years)	PRIMI		MULTI		CONTROL	
	N	%	N	%	N	%
≤ 20 Years	1	3.33	0	0	3	10.00
21 - 30 Years	27	90.00	20	66.67	18	60.00
31 - 40 Years	2	6.67	9	30.00	9	30.00
41 - 50 Years	0	0	1	3.33	0	0
TOTAL	**30**	**100**	**30**	**100**	**30**	**100**
Mean	25.67		30.13		27.63	
Standard Deviation (sd)	3.38		4.90		4.99	

The mean age of distribution of subjects taken up in the study is about 27 years. The mean age of the primi was 25 years and that of multi was 30 years. Age group of 20 to 30 years is fertile period and it's a high time to intervene.

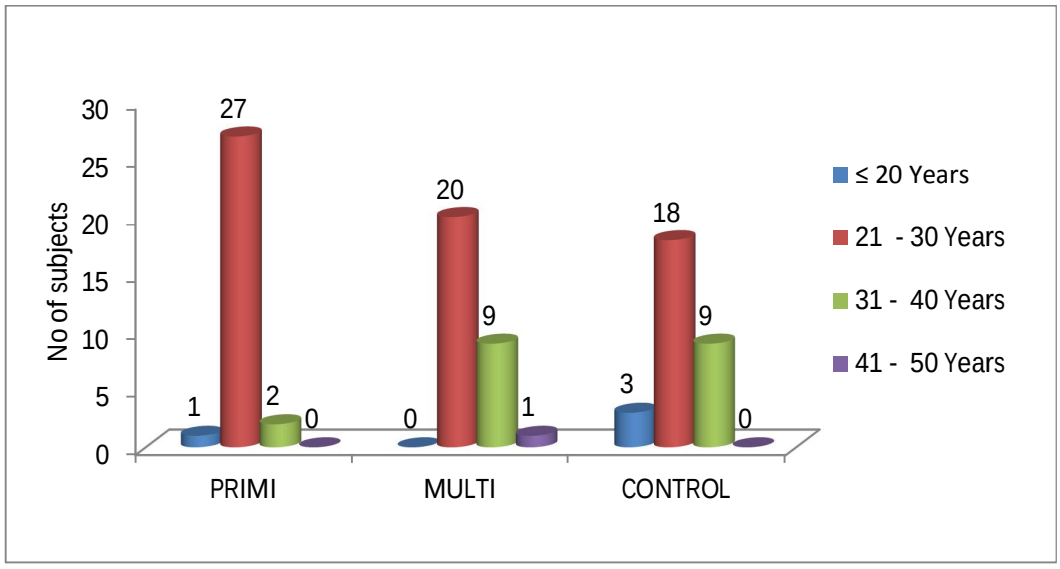

Fig. 9 : Bar diagram showing the age distribution among primi and mutiparous cases with the control.

TABLE-2 : Height distribution of study Subjects

Height (m)	CASE		CONTROL	
	Number	Percentage	Number	Percentage
≤ 1.50	33		16	
1.51 - 1.55	19		7	
1.56 - 1.60	7		5	
1.61 - 1.65	1		2	
TOTAL	**60**	**100**	**30**	**100**
Mean	1.51		1.51	
Standard Deviation (sd)	0.04		0.06	
t-value	0.11			
P-value	0.92			
Significant	Not Significant			

The mean height of the cases and controls included in the study was around 1.51 m. It was statistically not significant, on comparing the two groups.

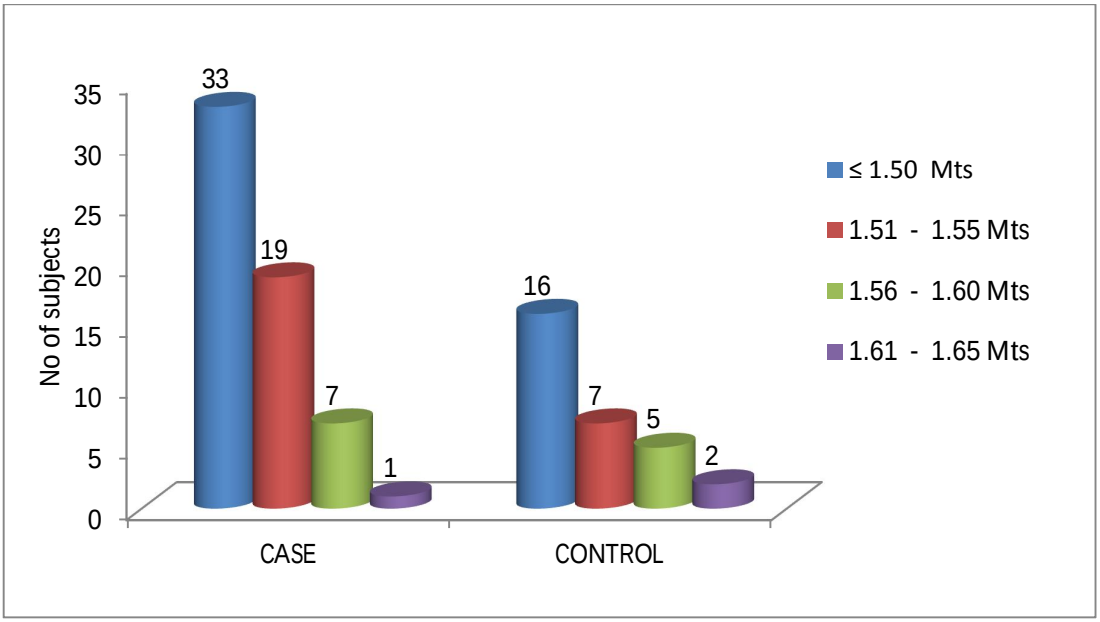

Fig 10: Bar diagram showing the distribution of height between the cases and control

TABLE-3 : Weight distribution of study Subjects

Weight (kg)	CASE		CONTROL	
	Number	Percentage	Number	Percentage
≤ 40 Kg	1		1	
41 – 50 Kg	5		3	
51 - 60 Kg	8		12	
61 - 70 Kg	18		8	
71 - 80 Kg	19		5	
81 - 90 Kg	7		1	
91 - 100 Kg	1		0	
≥101 Kg	1		0	
TOTAL	**60**	**100**	**30**	**100**
Mean	68.43		60.63	
Standard Deviation (sd)	12.81		10.52	
t-value	2.88			
P-value	0.01			
Significant	Significant			

The mean weight distribution among the cases was 68.43kg whereas that of the controls was only 60.63kg. The P value was 0.01, shows it to be statistically significant. The pre- gestational weight and the gain of weight during pregnancy should be compared, which is more significant.

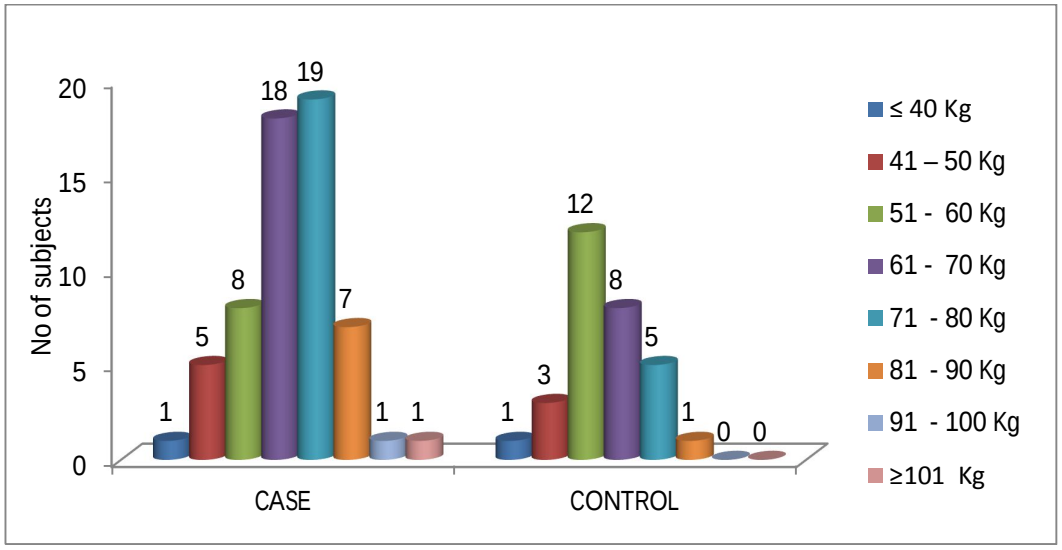

Fig 11: Bar diagram showing the distribution of weight distribution among the two groups.

TABLE-4 : BMI distribution of study Subjects

BMI (kg/m^2)	CASE		CONTROL	
	Number	**Percentage**	**Number**	**Percentage**
Under Weight	1		1	
Normal Weight	11		11	
Over Weight	20		10	
Obese	28		8	
TOTAL	**60**	**100**	**30**	**100**
Mean	29.92		26.53	
Standard Deviation (sd)	5.37		4.68	
t-value	2.93			
P-value	0.05			
Significant	Significant			

The mean BMI of the cases was 29.92 and that of the controls was 26.53. The P value was 0.05, which is statistically significant.

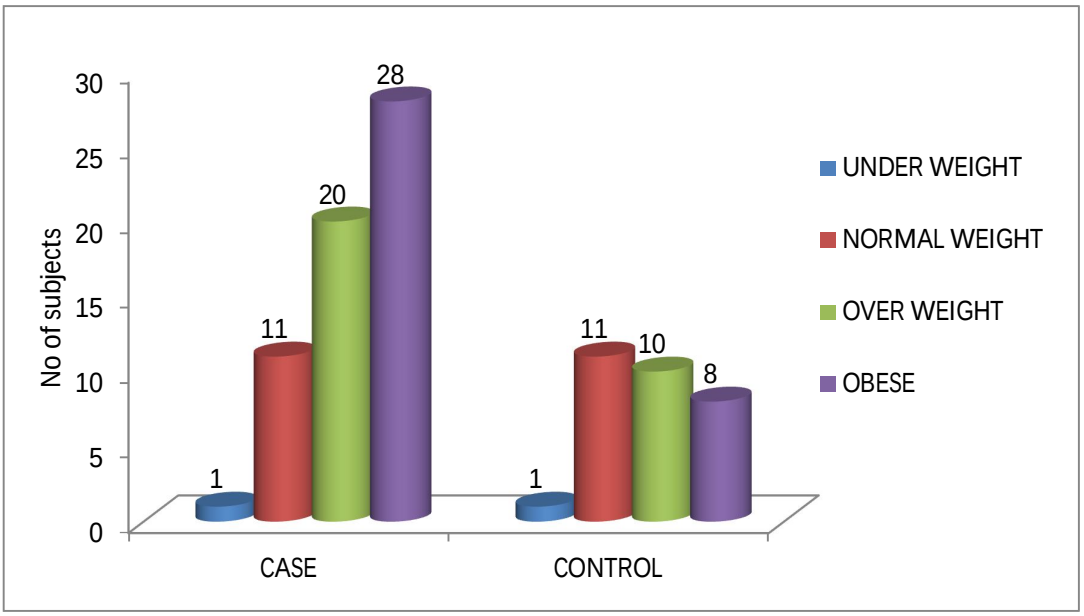

Fig 12: Bar diagram showing the distribution of BMI among the cases and controls

TABLE-5 : Family History of Diabetes

	CASE		CONTROL	
	Number	**Percentage**	**Number**	**Percentage**
NO	35	58.33	25	83.33
YES	25	41.67	5	16.67
TOTAL	**60**	**100**	**30**	**100**
Chi-square	5.63			
p-value	0.02			
Significant	Significant			

Family history of diabetes was significant among the cases when compared to the controls.

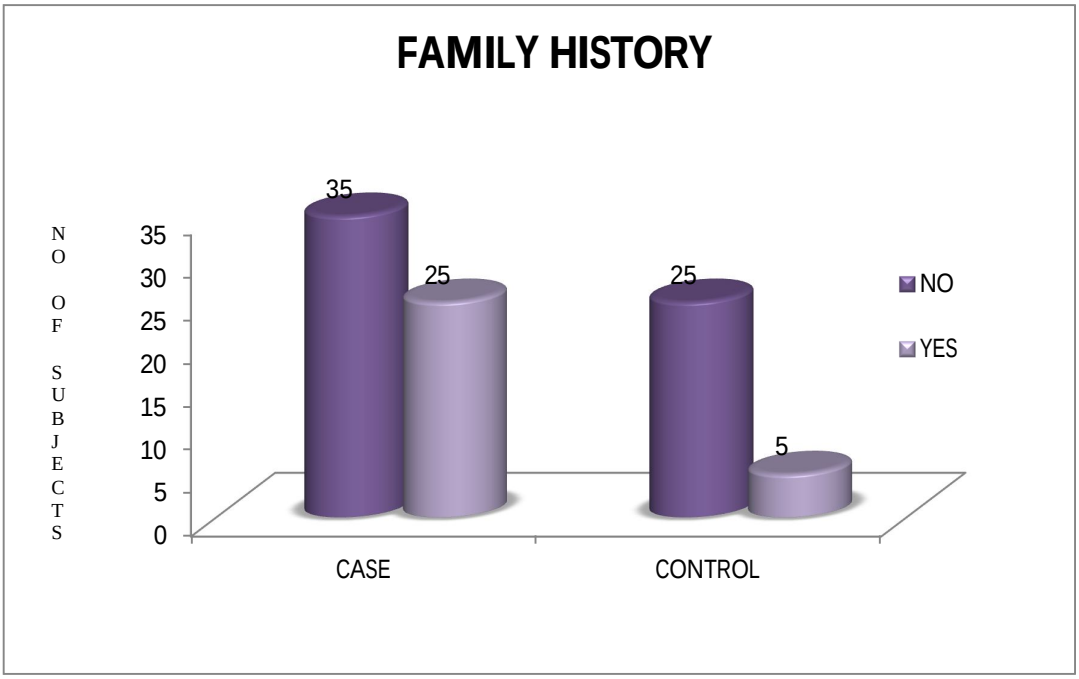

Fig 13: Bar diagram showing the association of family history with GDM

TABLE-6 : Previous History of GDM

	CASE		CONTROL	
	Number	**Percentage**	**Number**	**Percentage**
NO	51	85.00	30	100
YES	9	15.00	0	0
TOTAL	**60**	**100**	**30**	**100**
Chi-square	5.00			
p-value	0.03			
Significant	Significant			

On comparing the previous history of GDM between the multiparous women, it was significant among the cases.

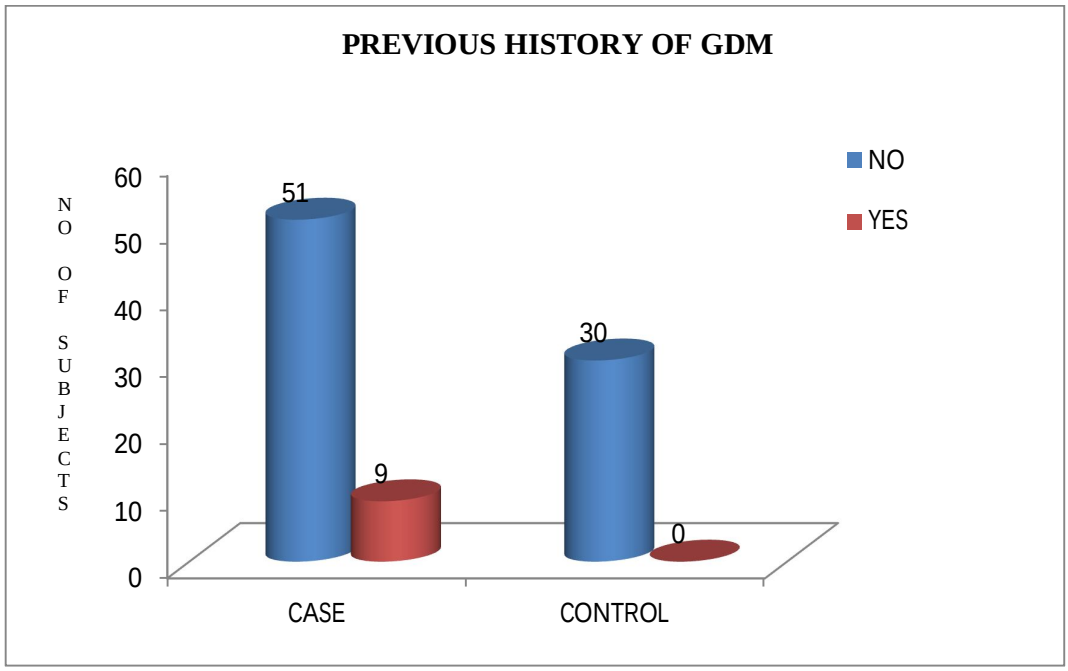

Fig 14: Bar diagram showing the distribution of previous history of GDM between cases and controls

TABLE-7 : FBS

FBS (mg/dL)	CASE		CONTROL	
	Mean	**Sd**	**Mean**	**sd**
FBS	107.78	19.02	75.83	13.27
t-value	8.24			
P-value	0.000			
Significant	Highly Significant			

The normal fasting blood glucose level is 70 to 100 mg/dL. The mean fasting blood sugar among the GDM patients was 107.78 mg/dL and that of the control group was only 75.83 mg/dL. In normal pregnant women, it indicates absolute control of blood glucose. The variation of blood glucose was statistically highly significant with a P value of <0.001.

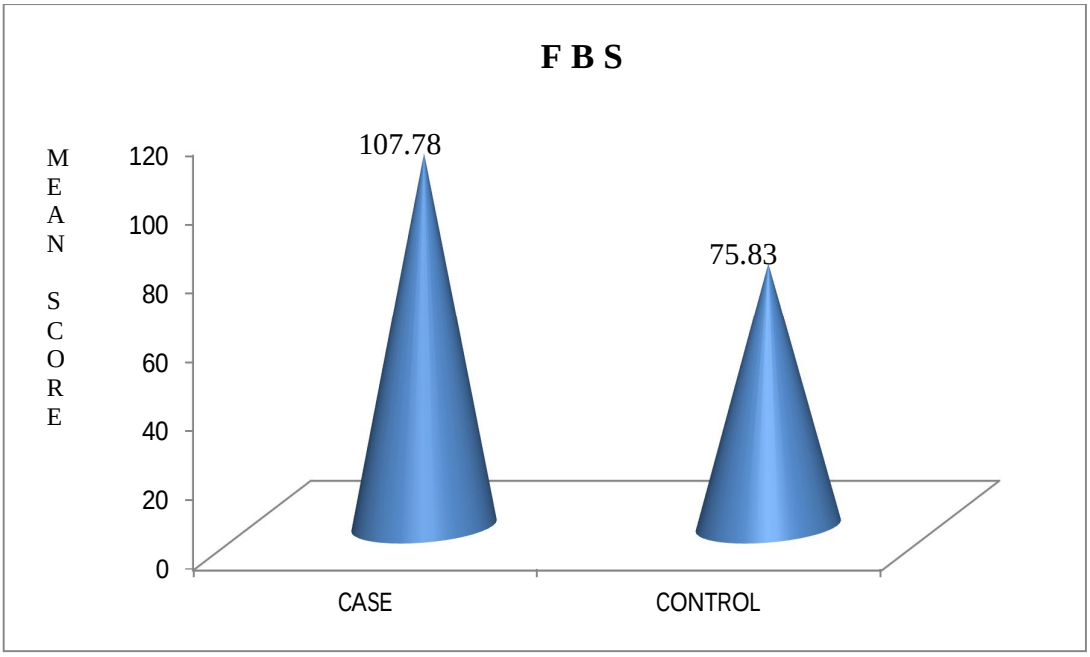

Fig 15: Bar diagram showing the distribution of FBS among the two groups

TABLE-8 : HbA_1c

HbA_1c (%)	CASE		CONTROL	
	Mean	**Sd**	**Mean**	**sd**
	6.52	0.90	5.11	0.53
t-value	7.88			
P-value	0.000			
Significant	Highly Significant			

A value of <5.7% is considered normal.The mean HbA_1c value in the GDM cases was 6.52% which varies from that of normal pregnant women with highly significant P value of <0.001.

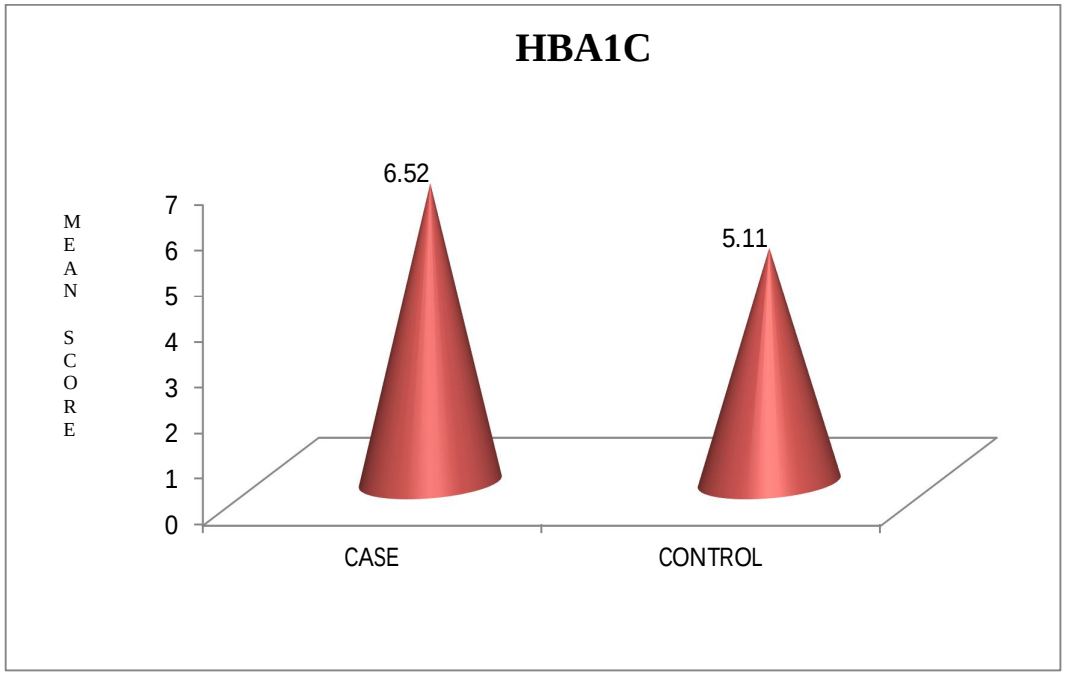

Fig 16: Bar diagram showing the distribution of HbA_1c levels between the cases and control groups

TABLE-9 : RBP4

RBP4 (mg/L)	CASE		CONTROL	
	Mean	**Sd**	**Mean**	**sd**
	50.56	12.62	22.71	5.87
t-value	11.46			
P-value	0.000			
Significant	Highly Significant			

According to previous studies, RBP4 levels of <40 mg/L during pregnancy was considered normal. In this study the mean RBP4 level in the pregnant women with GDM was 50.56 mg/L which was very high when compared to the mean in the control group, which was only 22.71 mg/L. It was shown to be statistically highly significant with a P value <0.001.

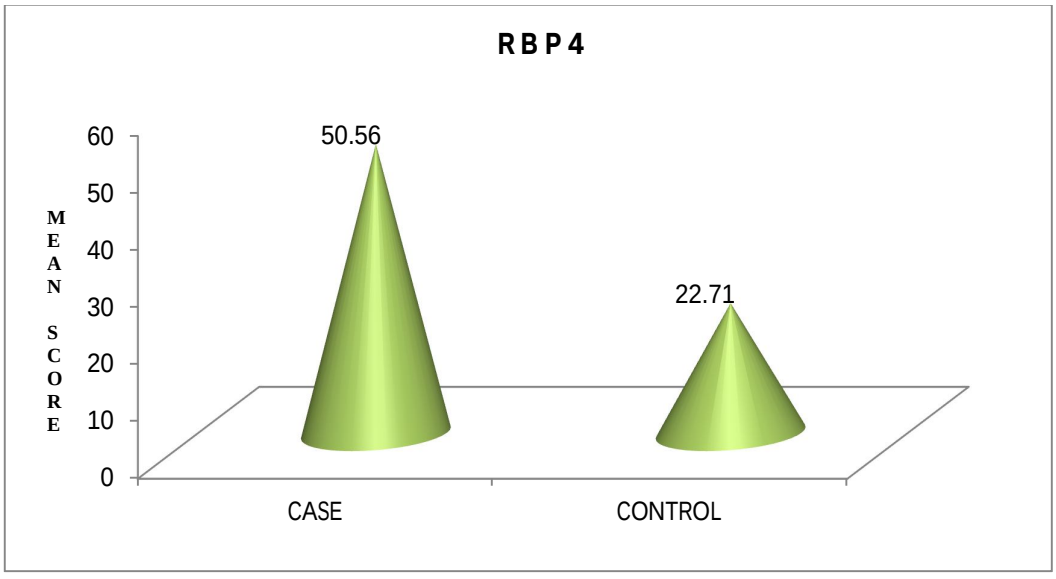

Fig 17: Bar diagram showing the distribution of RBP4 among the two groups

TABLE-10 : T.Cholesterol

T.Cholesterol (mg/dL)	CASE		CONTROL	
	Mean	**Sd**	**Mean**	**sd**
	226.23	34.93	208.47	38.21
t-value	2.21			
P-value	0.03			
Significant	Significant			

TABLE-11 : T_Cholesterol

T_Cholesterol (mg/dL)	**PRIMI**		**MULTI**		**CONTROL**	
	Mean	**Sd**	**Mean**	**Sd**	**Mean**	**sd**
	218.30	35.04	234.17	33.52	208.47	38.21
F-value	3.97					
P-value	0.02					
Significant	Significant					

The desirable cholesterol level is <200 mg/dL. The mean T.Cholesterol level in the GDM cases was 226.23 md/dL, which shows significant elevation in primi and multi, when compared to the control group. It was shown to have a P value of 0.03 which is statistically significant.

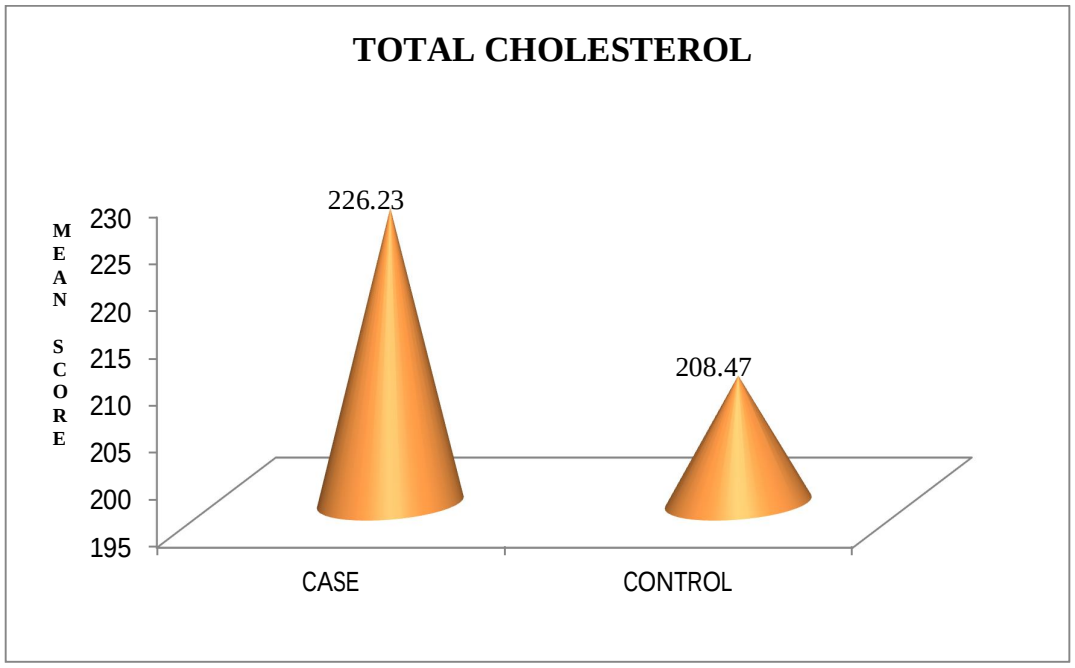

Fig 18: Bar diagram showing the distribution of T.Cholesterol among the two groups.

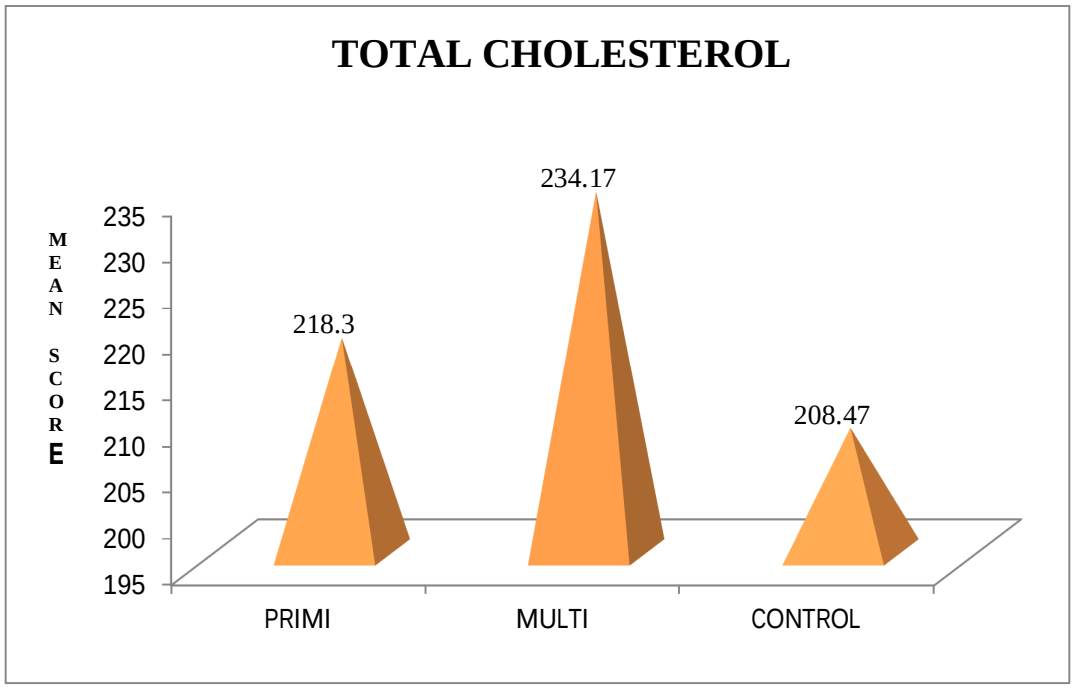

Fig 19: Bar diagram showing the distribution of T.Cholesterol in primi, multi and control groups.

TABLE-12 : TGL

TGL (mg/dL)	CASE		CONTROL	
	Mean	**Sd**	**Mean**	**sd**
	262.25	43.52	217.53	60.32
t-value	4.03			
P-value	0.001			
Significant	Highly Significant			

TABLE-13 : TGL

TGL(mg/dL)	**PRIMI**		**MULTI**		**CONTROL**	
	Mean	**Sd**	**Mean**	**Sd**	**Mean**	**sd**
	256.47	45.29	268.03	41.64	217.53	60.32
F-value	8.49					
P-value	0.000					
Significant	Highly Significant					

The normal reference range is <150 mg/dL. The mean triglyceride level in the cases was 262.25 mg/dL and that in the control pregnant women was 217.53 mg/dL. It was statistically highly significant with a P value of 0.001. When the primi were compared with multi and control, we got an F value of 8.49 and the P value was <0.001, which is statistically highly significant.

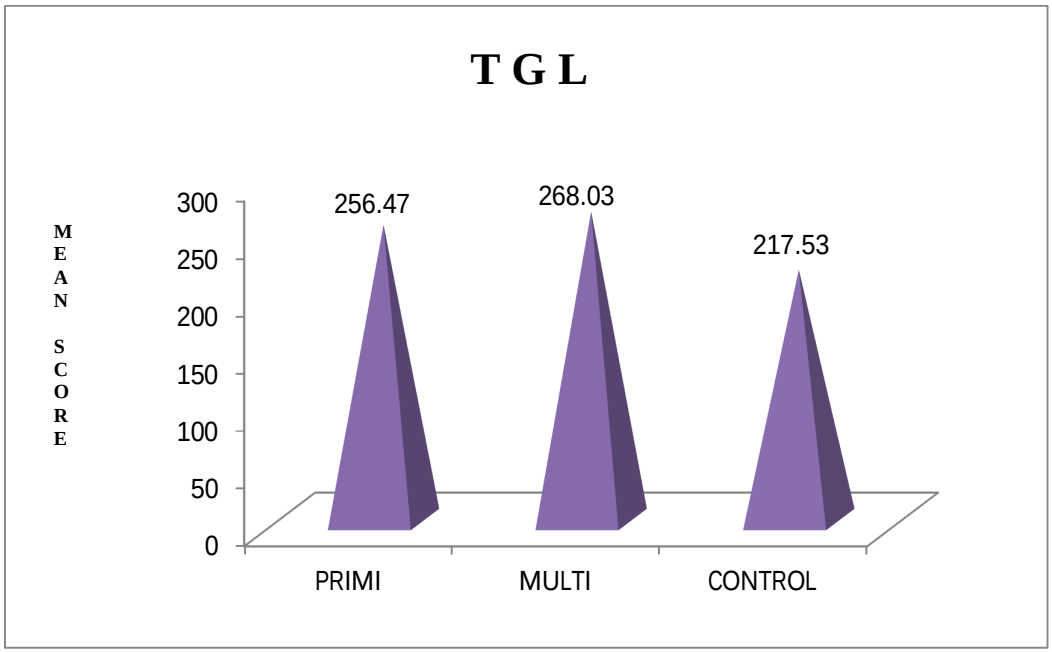

Fig 20: Bar diagram showing the distribution of triglycerides in GDM patients-primi and multi and controls.

TABLE-14 : HDL

HDL (mg/dL)	**CASE**		**CONTROL**	
	Mean	**Sd**	**Mean**	**sd**
	45.65	8.83	48.07	13.55
t-value	1.02			
P-value	0.31			
Significant	Not Significant			

>50 mg/dL is considered normal for females. On comparing the HDL values between the two groups, the cases had a mean of 45.65 mg/dL and control had 48.07 mg/dL. The difference was statistically not significant.

TABLE-15 : LDL

LDL (mg/dL)	CASE		CONTROL	
	Mean	**Sd**	**Mean**	**sd**
	128.13	32.83	116.89	34.65
t-value	1.50			
P-value	0.14			
Significant	Not Significant			

The mean LDL among the GDM patients was 128.13 mg/dL and that in control group was 116.89 mg/dL, which was statistically not significant.

TABLE-16 : Correlation with RBP4

	CASE		CONTROL	
	r	**P-value**	**r**	**P-value**
TGL	0.46	0.000**	-0.40	0.03*
HbA1c	0.44	0.000**	0.38	0.04*

Note : ** Correlation is Significant at 0.01 * Correlation is Significant at 0.05

Correlation analysis showed that Sr.RBP4 level were as high as that of triglyceride (r=0.46) with a P value of < 0.001 which was highly significant. Sr.RBP4 levels also positively correlated with HbA1c levels (r=0.44) with a P value of < 0.001, which is statistically highly significant.

TABLE - 17 : STEPWISE LINEAR REGRESSION ANALYSIS OF INDEPENDENT PREDICTORS OF RBP4

Unstandardized Coefficients			
Variables	**B**	**T**	**p-value**
BMI	0.039	0.606	0.545
FBS	0.148	1.866	0.064
T.CHOLESTRAL	0.067	1.033	0.303
HDL	0.093	1.476	0.142
LDL	0.094	1.532	0.127
HB	0.111	1.784	0.076
HbA1c	7.675	7.348	**0.000**
TGL	0.079	3.911	**0.000**

Stepwise linear regression analyses revealed that HbA_1c and triglycerides (r^2 =0.279) were independent predictors of RBP4.

Discussion

DISCUSSION

This study on Serum RBP4 levels in pregnant women with gestational diabetes mellitus was taken up and done as 3 groups with primiparous, multiparous and normal pregnant women as control group in the age group of 21 to 40 years.

The prevalence of gestational diabetes mellitus (GDM) has increased by 10-100% in the past 20 years[119]. Apart from its adverse effects on infants during the newborn period ,it also contributes to the current trend of increasing obesity and diabetes[120].In the study done by Coustan DR et al, it was shown that the prevalence of GDM usually reflects the frequency of type 2 diabetes in that population[121,122]. The risk factors for Gestational diabetes mellitus are obesity, advanced maternal age, family history of diabetes and ethnicity.

In India, Gestational diabetes is more common in urban women than those living in rural areas[123].

The major contributing factors for the increase in prevalence in developing countries are

1) Trend towards increasing maternal age
2) Epidemic of obesity
3) Decrease in physical activity[124] and
4) Adoption of modern lifestyle[125].

Relatively only a few published studies are available regarding the genetic susceptibility of gestation diabetes. The available data suggest that GDM has a familial tendency. There is a tendency for GDM to recur in atleast 30% of women with previous history. These suggest that GDM develops in those who are genetically predisposed.

This case control study has been undertaken to estimate serum Retinol binding protein-4 (RBP4) levels in patients with gestational diabetes mellitus and to correlate its level with lipid profile and HbA1c levels.

RBP4, secreted by liver and the adipocytes, is a new adipokine which has been shown to contribute to insulin resistance .Serum RBP4 levels have already shown to be elevated in obesity and Type 2 diabetes. These suggest that the correlation between levels of Sr.RBP4 and insulin resistance has been consistent. But the data about Sr.RBP4 levels in pregnant women are limited. So the present study was taken up to show the association of RBP4 with GDM.

We included pregnant women in the age group of 21 to 40 years. The cases and controls were selected based on their OGTT results which they undergo routinely during prenatal examination.

Adipose tissue is recognized as an endocrine organ. The expansion of adipose tissue due to obesity triggers chronic low grade inflammation. In study done by Clement,k; Viguerie and Cancello, R; Henegar has shown that weight loss decreases macrophage infiltration and expression of pro- inflammatory cytokines in adipose tissue[116].

Table -3 and 4 shows the distribution of weight and BMI among the cases and control. There was statistically significant difference in the distribution of weight and BMI between the cases and control group. The mean weight of the gestational age matched pregnant women with GDM was 68.43kg and those without GDM was 60.63kg. Also the BMI of the cases and control women showed significant difference. The mean BMI of cases was 29.92 and that of controls was 26.53 with a P-value of 0.05.

These values signifies the association of obesity as one of the underlying pathology in the development of GDM. In the Study done by Bhartha JL et al,it was found that, measurement of visceral adiposity correlates better than subcutaneous fat or BMI with insulin resistance. In our study, BMI was shown to be a significant indicator.

Gestational diabetes mellitus itself is characterized by amplification of low-grade inflammation. This is supported by the increased levels of pro-inflammatory cytokines like TNFα and IL-6 in GDM. It is also known that obesity is strongly associated with inflammation, which contributes to insulin resistance of GDM.

Table 5 shows the comparison of family history between the cases and control. Table 6 shows the comparison of previous history of GDM among the multiparous women. This study shows that, family history of T2DM and previous history of GDM was significantly more prevalent in the GDM group with a P value of 0.02 and 0.03 respectively. This clearly indicates the genetic predisposition in the etiopathogenesis of GDM. This is supported by a previous

study in Danish twins by Poulsen et al in 2005. It was shown that both the twins showed major genetic components in both traits. In study done by Lambrinoudaki I et al showed that more than 75% variation in insulin secretion trait and 53% of peripheral insulin sensitivity are explained by genetic components[117].

GDM develops when the genetic predisposition of pancreatic islet β-cell impairment is unmasked by the increased insulin resistance during pregnancy[118].

Table 7 and 8 shows the comparison of fasting blood glucose levels and HbA_1c between the cases and controls. The serum fasting blood glucose and HbA_1c levels were significantly higher in the GDM group. Their P value in comparison to the control group was highly significant.

Normally, during pregnancy, there is decrease in fasting blood glucose level compared to the non- pregnant women due to

1) The anabolic effect of insulin
2) Increase in plasma volume
3) Increase in feto-placental glucose utilization
4) Decrease in substrates for gluconeogenesis, mainly alanine.

The target HbA_1c value to be achieved in GDM is 6%. But study done by Carr S et al and Coustan DR et al has shown that measurement of HbA_1c is less sensitive in GDM. But in this study HbA1c was shown to be significantly correlated with GDM patients.

Tables 10 to 15 shows the comparison of lipid profile (TC,TGL,HDL and LDL) among the cases and controls. On comparing the lipid profile values

between the cases and control, there was significantly high value of total cholesterol and triglyceride levels in cases with a statistically significant P value of 0.03 for total cholesterol and highly significant P value of 0.001 for triglycerides. There was also statistically highly significant P value of <0.001 by on comparing as 3 groups as primi vs multi vs control group.

But the HDL cholesterol levels and LDL cholesterol levels between the two groups were statistically not significant.

In study done by Rafael Carmena, it was shown that in diabetes there is elevated triglycerides and low HDL levels. The increased TGL drives the exchange of core lipids between triglyceride rich lipoprotein and HDL particles. There is increased cholesterol ester transfer protein mediated transfer of cholesterol esters to VLDL and also transfer of TGL to HDL. Triglycerides in HDL are substrate for hepatic lipase and its hydrolysis produces smaller HDL particles and free apoA1. These small HDL particles in circulation are rapidly metabolised than the normal HDL, resulting in reduced HDL levels. Thus, HDL enrichment with TGL as well as increased hepatic lipase activity plays a basic role in lowering plasma HDL levels.

Various studies have already shown the worsen state of dyslipidemia in GDM. In the study done in the Shanghai Jiaotong University in a cross sectional study between GDM and normal pregnant women, it was shown that total cholesterol and triglycerides were elevated in both the groups when compared to non-pregnant women.

The altered lipid metabolism is common in normal pregnancy with significantly elevated lipids, especially TGL. In GDM, the physiological changes in insulin and lipid levels are exaggerated, indicating a state of underlying metabolic dysfunction that manifests during pregnancy.

The main characteristics of Diabetes mellitus is:

Elevated TGL, lowered HDL and raised small dense LDL levels, which forms the atherogenic triad.

Insulin resistance is associated with excessive flux of free fatty acids for VLDL synthesis. Other component includes elevated apoB and prolonged post prandial lipemia.

Obese pregnant women accumulate nearly the same amount of fat as lean women, but it is more of a central distribution. This reflects their insulin resistant state. Obese women are dyslipidemic with elevated TG and VLDL and reduced HDL levels. This results in increased oxidative stress caused by the accumulated lipid in the endothelium. This stands as a link between obesity and increased risk of adverse outcomes during pregnancy, including pulmonary embolism. Impairment of lipid metabolism is a risk factor for cardiovascular diseases.

Peroxisome proliferator- activated receptor gamma (PPARγ), which is a nuclear hormone receptor, is mainly expressed in adipocytes. It plays a key role in the ability of adipose tissue to buffer fatty acid influx. GDM may be associated with dominant negative loss of function mutations in the ligand binding site of PPARγ, which manifests as a stereotyped form of partial lipodystrophy and

impaired postprandial fatty acid trapping. They may also have fatty liver and are severely insulin resistant.

Table 9 shows the comparison of serum RBP4 levels between the patients with GDM and control pregnant women. Serum RBP4 levels were significantly elevated in GDM with a statistically highly significant P value of <0.001. This is consistent with the results obtained in various studies including the one by Arash Hossein-Nezhad et al. But in our study the association between RBP4 and HOMA-IR was not done.

In study done by Chan et al and colleagues, found higher RBP4 levels in GDM but the levels of RBP4 did not correlate with insulin resistance. But in a study by Choi et al colleagus, it was stated that a significant high RBP4 levels in women with previous history of GDM were related with insulin resistance.

This study does not agree with the results of the study done by Ueland et al and colleagues which reported low level of RBP4 in women with GDM, which could be due to different levels of BMI.

RBP4 (Retinol Binding Protein-4) is secreted by adipocyte which is elevated even before the development of frank diabetes. Serum RBP4 is elevated in obesity as well as in type 2 diabetes mellitus, and is one of the main contributor of insulin resistance.

It has been shown in previous studies that RBP4 in pregnancy strongly correlates with insulin resistance (fasting insulin and HOMA-IR). Our present finding of markedly elevated RBP4 levels in GDM suggest that, it may be an

early marker in the natural history of T2DM with potential implications for the screening and prevention of the disease. But BMI matched strategy is more credible.

In the study done by Choi et al and colleagues, if was found that RBP4 levels after pregnancy in women with previous history of GDM correlated positively with systolic blood pressure, abdominal fat as well as fasting insulin concentration. It was also reported that, RBP4 levels independently correlates with fasting plasma glucose and triglyceride levels in women with previous history of GDM.

RBP4 are elevated because of increase in size of the adipocytes. RBP4 is a pathophysiological link between obesity and insulin resistance. Studies have demonstrated the role of RBP4 in lipid metabolism and metabolic syndrome. Von Eynatten et al. has also found significant correlation between RBP4 and LDL cholesterol, VLDL triglycerides and activity of hepatic lipase in patients with type 2 diabetes and cardiovascular disease.

In the study done by Arash et al, it was stated that 32.6% of GDM patients had metabolic syndrome after pregnancy, which is very high compared to healthy pregnant women, where it was only 10.5%. They also found that , gestational RBP4 concentrations in 69.6% of women with metabolic syndrome in the postpartum period was more than 42 mg/mL(P value of 0.011) This level of RBP4 could predict GDM independent of age and BMI by logistic regression model.

Table 16 shows the correlation between RBP4 with triglycerides. It shows that RBP4 levels correlates positively with triglyceride, with statistically highly significant P value of <0.001. It also shows that RBP4 levels correlates positively with HbA1c levels, with statistically highly significant P value of <0.001.

One of the major consequences of insulin resistance on lipid metabolism loss of suppression of mobilisation of fat from adipose tissue by insulin thus increasing FFA levels. In many studies including the one done by Baynes et al on the role of hepatic and lipoprotein lipase in T2DM have shown the key role of insulin resistance (where there is suppressed LPL activity and elevated HPL activity) in causing hypertriglyceridemia. Thus in our study, RBP4 which is a marker of insulin resistance, correlates with the elevated levels of TGL.

In study done by Axelsen et al, it has been shown that abnormal clearance of triglyceride rich lipoproteins in the post-prandial phase is an early defect in T2DM, since healthy first degree relatives of patients with T2DM exhibit postprandial hypertriglyceridaemia despite having normal fasting triglyceride levels. Thus Sr.RBP4 which correlates with TGL levels, could be used as an early marker of diabetes in pathogenic and pre-pathogenic phase itself.

This indicates that, when there is an increase in RBP4 level, there is an associated rise in triglyceride levels as well as HbA_1c levels. This shows that RBP4 is directly linked with insulin resistance, as it influences both carbohydrate and lipid metabolism in those patients with high values.

Table 17 shows the stepwise linear regression analysis of independent predictors of serum retinol binding protein 4 (RBP4) in GDM. Stepwise linear regression analysis has revealed that triglycerides and HbA_1c were independent predictors of serum RBP4 concentrations in pregnant women.

Studies have shown that elevated level of RBP4 indicates the chance for developing type 2 diabetes mellitus after pregnancy. It is proven that GDM women have 17% to 63% of increased risk of developing T2DM in the next 5 to 16 years. 23% of Iranian women with history of GDM were shown to develop frank diabetes or impaired glucose tolerance by 6 to 12 weeks after delivery.

It should also be noted that GDM increases the risk of hypertension, susceptibility to atherosclerosis and coronary artery disease (CAD). Persistent hyperinsulinemia causes raised levels of Sr.triglycerides, free fatty acids and LDL. It also decreases the HDL levels. Elevated free fatty acid levels in the blood causes activation of the innate immune system to release pro- inflammatory cytokines like TNF- α, IL-6 and IL-1β. These cytokines leads to alteration in insulin sensitivity resulting in disruption of glucose homeostasis.

Initially there is insulin signaling by these cytokines in liver, muscle and adipose tissue. There is decrease in the functioning liver X receptors (LXRs) as well as increased cholesterol accumulation. These act as a stimulant for the increased hepatic release of inflammatory markers like CRP, Plasminogen inhibitor -1, α1- acid glycoprotein and haptoglobin. They also stimulate fibrinogen, adding up to cause coronary artery disease.

Cytokines also increases VLDL and FFA production, resulting in diabetic dyslipidemia and increased plaque accumulation.

Retinol binding protein -4 is shown to be elevated in the serum before the development of frank diabetes. It serves as an important tool to identify insulin resistance as well as the cardiovascular risk in these subjects. These findings provide a rationale for choosing anti-diabetic therapies aimed to lower serum RBP4 levels.

Our present study suggests that higher levels of RBP4 in GDM may be considered as an early marker in the natural history of type 2 diabetes mellitus, which may have potential implications for screening and also in the prevention of the disease.

Summary & Conclusion

SUMMARY AND CONCLUSION

A study on estimating serum RBP4 levels in GDM was taken up. The study was conducted in three groups in the age group of 21 to 40 years

A) Primiparous women with GDM

B) Multiparous women with GDM

C) Normal pregnant women as controls

From this study we conclude that:

1. Serum retinol binding protein 4 (RBP4) levels are higher in patients with gestational diabetes mellitus compared to the control group with statistically highly significant P-value of <0.001.
2. RBP4 levels correlates with increase in triglycerides and vice versa, that is there is highly significant correlation.
3. Rise in RBP4 level correlates with rise in HbA_1c levels, showing a highly significant correlation.
4. Mean fasting blood glucose levels are significantly higher among the GDM patients compared to the control group.
5. There is elevated total cholesterol and triglyceride levels among the GDM patients compared to the control group.
6. Family history is significant among the cases when compared to the control group.
7. Previous history of GDM is significant in patients with GDM in present pregnancy.

Limitations of the study

LIMITATIONS OF THE STUDY

The following were the limitations of this study

1. In our study serum RBP4 was measured by ELISA method. Measurement by Western blot technique is the gold standard for determination of RBP4.
2. RBP4 is bound to both retinol and transthyretin in plasma. So measuring these would have been more useful. But it is an elongated procedure.
3. The present study did not measure fasting insulin level to directly determine the HOMA-IR levels. A measure of HOMA-IR directly indicates insulin resistance. If this was also taken up and done in our present study and correlated with RBP4 levels, then it will still show that RBP4 is a marker for insulin resistance directly. But in our present study RBP4 as marker of insulin resistance has been shown indirectly.

Scope for further studies

SCOPE FOR FURTHER STUDIES

1. Study on effects of RBP4 concentration on sex hormones.
2. Study on RBP4 levels in postpartum period in mother with GDM and its correlation with insulin resistance.
3. Study on RBP4 levels in cord blood of neonates born to GDM mothers.
4. Correlation of RBP4 with C-peptide levels in patients with T2DM and GDM.
5. Study on RBP4/ apo RBP4 levels in blindness.
6. Study on correlation between serum RBP4 levels and cataract.
7. A study on RBP4 levels in short stature primi with age >30 years.
8. Study on RBP4 levels in acne and its variations with retinoid treatment.
9. A comparative study between RBP4 and leptin in GDM.
10. A correlation study between RBP4 and insulin receptors.
11. Effect of reduction of TGL levels with drugs on the levels of RBP4.
12. RBP4 can also be used as a marker for subclinical hypothyroidism and also for diagnosis of Non alcoholic fatty liver disease (NAFLD).

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Annexures

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI 600 003

EC Reg.No.ECR/270/Inst./TN/2013
Telephone No.044 25305301
Fax: 011 25363970

CERTIFICATE OF APPROVAL

To
Dr. R.Gayathri
Post Graduate in M.D. - Bio chemistry
Institute of Biochemistry,
Madras Medical College, Chennai-3

Dear ,

The Institutional Ethics Committee has considered your request and approved your study titled **"SERUM RETINOL BINDING PROTEIN 4(RBP4) LEVEL INPATIENTS WITH GESTATIONAL DIABETES MELLITUS " NO.02092016** .

The following members of Ethics Committee were present in the meeting hold on **06.09.2016** conducted at Madras Medical College, Chennai 3

1. Prof. C. Rajendran, MD.	Chairperson
2. Prof. Dr. M.K. Muralidharan, M.S, M.Ch., MMC ,Ch-3	Deputy Chairperson
3. Prof. Sudha Seshayyan, MD., Vice Principal, MMC.Ch- 3.	Member Secretary
4. Prof. B.Vasanthi,MD.,Prof of Pharmacology, MMC,	Member
5. Prof. P.Raghumani.MS., Professor of Surgery, Inst. of surgery	Member
6. Prof. R.Padmavathy,MD., Professor, Inst.of Pathology, MMC,Ch	Member
7. Tmt.J.Rajalakshmi, Junior Administrative Officer,MMC,Ch	Layperson
8. Thiru.S.Govindasamy., B.A.B.L., High Court, Chennai-1	Lawyer
9. Tmt.ArnoldSaulina, MA., MSW.,	Social Scientist

We approve the proposal to be conducted in its presented form.

The Institutional Ethics Committee expects to be informed about the progress of the study and SAE occurring in the course of the study, any changes in the protocol and patients information/informed consent and asks to be provided a copy of the final report.

Member Secretary - Ethics Committee

MEMBER SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE
CHENNAI-600 003

MADRAS MEDICAL COLLEGE * CHENNAI-600 003 *

21 SEP

PROFORMA

NAME: AGE: Patient ID No:

PARITY: GESTATIONAL AGE:

HEIGHT: WEIGHT: BMI:

OGTT VALUES:

FAMILY H/O T2DM:

PREVIOUS H/O GDM: PREVIOUS H/O CEASAREN:

H/O THYROID DISORDER: YES/NO

H/O HYPERTENSION: YES/NO

H/O CORONARY HEART DISEASE: YES/NO

H/O OTHER MEDICAL ILLNESSES: YES/NO

Drug history:

THYROID MEDICATIONS: YES/NO

LIPID LOWERING DRUGS YES/NO

ORAL HYPOGLYCAEMIC AGENTS YES/NO

ANY OTHER MEDICATIONS:

INVESTIGATIONS:

FASTING PLASMA GLUCOSE

LIPID PROFILE : SERUM TGL,SERUM

TOTAL CHOLESTEROL

SERUM HDL-CHOLESTEROL

HBA_1C LEVEL.

SERUM RBP4 LEVEL.

INFORMATION SHEET

- Your blood sample has been accepted.
- We are conducting a study on patients with gestational diabetes mellitus at Rajiv Gandhi Government General Hospital, Chennai and for that your blood sample may be valuable to us.
- The purpose of this study is to assess level of serum retinol binding protein 4 (RBP4) in patients with gestational diabetes mellitus with the help of certain special tests.
- We are selecting certain cases and if your blood sample is found eligible, we may be using your blood sample to perform extra tests and special studies which in any way do not affect your final report or management.
- The privacy of the patients in the research will be maintained throughout the study. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.
- Taking part in this study is voluntary. You are free to decide whether to participate in this study or to withdraw at any time; your decision will not result in any loss of benefits to which you are otherwise entitled.
- The results of the special study may be intimated to you at the end of the study period or during the study if anything is found abnormal which may aid in the management or treatment.

Signature of investigator Signature of participant

Date:

PATIENT CONSENT FORM

Title of the study **: SERUM RETINOL BINDING PROTEIN (RBP4) LEVEL IN WOMEN WITH GESTATIONAL DIABETES MELLITUS .**

Name : Date :

Age :

GESTATIONAL AGE: OP No :

Project Patient No :

The details of the study have been provided to me in writing and explained to me in my own language.

I confirm that I have understood the above study and had the opportunity to ask questions.

I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected.

I agree to use my personal clinical history and investigation details for the purpose of the study.

I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).

I have been given an information sheet giving details of the study.

Having understood _____d/o or w/o_____ give my consent to participate in the study conducted by DR.R.GAYATHRI, Post graduate, Institute of Biochemistry, Madras Medical College, Chennai.

Signature of the investigator: Signature of the participant

Place: Thump impression.

Date:

ஆராய்ச்சி தகவல் தாள்.

தங்களது இரத்தம் இங்குபெற்றுக்கொள்ளப்பட்டது.

சென்னை அரசு பொது மருத்துவமனையில் கர்ப்பகால நீரிழிவு நோயினால் பாதிக்கப்பட்ட பெண்களிடையே இரத்தத்தில் ரெட்டினால் பையிண்டிங் பிரோடின் 4 (RBP4) புரத அளவை மதிப்பிடுதல் பற்றிய ஆராய்ச்சி என்ற தலைப்பில் ஆராய்ச்சி நடைபெற்று வருகின்றது.

நீங்களும் இந்த ஆராய்ச்சியில் பங்கேற்க நாங்கள் விரும்பிகிறோம் .

இந்த ஆராய்ச்சியில் உங்களுடைய இரத்தம் எடுத்து சிறப்புப்பரி சோதனைக்கு உட்படுத்தி அதன் தகவல்களை ஆராய்வோம்.

அதனால் தங்களது நோயின் ஆய்வறிக்கையோ அல்லது சிகிச்சையோ பாதிப்புக்கு உள்ளாகாது என்பதையும் தெரிவித்துக்கொள்கிறோம்.

முடிவுகளை அல்லது கருத்துக்களை வெளியிடும் போதோ அல்லது ஆராய்ச்சியின் போதோ தங்களது பெயரையோ அல்லது அடையாளங்களையோ வெளியிடமாட்டோம் என்பதையும் தெரிவித்துக்கொள்கிறோம்

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களுடைய விருப்பத்தின் பேரில் தான் இருக்கிறது.மேலும் நீங்கள் எந்நேரமும் இந்த ஆராய்ச்சியிலிருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துகொள்கிறோம்.

இந்த சிறப்பு பரிசோதனைகளின் முடிவுகளை ஆராய்ச்சியின் போது அல்லது ஆராய்ச்சியின் முடிவின் போது தங்களுக்கு அறிவிப்போம் என்பதையும் தெரிவித்துக்கொள்கிறோம்.

ஆராய்ச்சியாளர் கையொப்பம் பங்கேற்பாளர் கையொப்பம்

தேதி:

நோயாளியின்ஒப்புதல்படிவம்

ஆராய்ச்சி தலைப்பு: கர்ப்பகால நீரிழிவு நோயினால் பாதிக்கப்பட்ட பெண்களிடையே இரத்தத்தில் ரெட்டினால் பையிண்டிங் பிரோடின் 4 (RBP4) புரத அளவை மதிப்பிடுதல் பற்றிய ஆராய்ச்சி.

பெயர் : தேதி :

வயது : புறநோயாளிஎண்:

கர்பகாலம் / கரு ஆயுட்காலம் : ஆராய்ச்சி சேர்க்கை எண் :

இந்த ஆராய்ச்சியின் விவரங்களும் அதன் நோக்கங்களும் முழுமையாக எனக்கு தெளிவாக விளக்கப்பட்டது.

எனக்கு விளக்கப்பட்ட விஷயங்களை புரிந்து கொண்டு நான் எனது சம்மதத்தைத் தெரிவிக்கிறேன்.

இந்த ஆராய்ச்சியில் பிறரின் நிர்பந்தமின்றி என் சொந்த விருப்பத்தின் பேரில் தான் பங்கு பெறுகிறேன் மற்றும் நான் இந்த ஆராய்ச்சியிலிருந்து எந்நேரமும் பின்வாங்கலாம் என்பதையும், அதனால் எந்த பாதிப்பும் ஏற்படாது என்பதையும் புரிந்து கொண்டேன் நான் கர்ப்பகால நீரிழிவு நோயினால் பாதிக்கப்பட்ட பெண்களிடையே இரத்தத்தில் ரெட்டினால் பையிண்டிங் பிரோடின் 4 (RBP4) புரத அளவை மதிப்பிடுதல் பற்றிய ஆராய்ச்சி என்ற தலைப்பில் மேற்கொள்ளப்படும் இந்த ஆராய்ச்சியின் விபரங்களைக் கொண்ட தகவல் தாளைப் பெற்றுக் கொண்டேன்.

இதன் மூலம் எந்த பின்விளைவும் வராது என மருத்துவர் மூலம் தெரிந்து கொண்டு என்னுடைய சுயநினைவுடன் மற்றும் முழு சுதந்திரத்துடன் இந்த மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக்கொள்ள சம்மதிக்கிறேன்.

தேதி கையொப்பம்

URKUND

Urkund Analysis Result

Analysed Document: RBP4 IN GDM.docx (D30362498)
Submitted: 9/4/2017 1:18:00 PM
Submitted By: gaya3_av@yahoo.co.in
Significance: 2 %

Sources included in the report:

Doddigarla Zephy (GH2589)- Thesis.docx (D19800274)
https://helda.helsinki.fi/handle/10138/22880
http://europepmc.org/articles/PMC2561276

Instances where selected sources appear:

5

PLAGIARISM CERTIFICATE

This is to certify that this dissertation work titled **"SERUM RETINOL BINDING PROTEIN 4 (RBP4) LEVEL IN PATIENTS WITH GESTATIONAL DIABETES MELLITUS"** of the candidate **DR.R.GAYATHRI** with registration Number **201523001** for the award of **M.D** in the branch of **BIOCHEMISTRY**. I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **2 percentage** of plagiarism in the dissertation.

Guide & Supervisor sign with Seal.